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Examination of Potato Virus X Subgenomic RNAs: Their Number, Their Coding Properties, and Their Possible Encapsidation.

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EXAMINATION OF POTATO VIRUS X SUBGENOMIC RNAS: THEIR NUMBER, THEIR CODING PROPERTIES, AND THEIR POSSIBLE ENCAPSIDATION

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ABSTRACT

Potato Virus X is the type member of the Potexvirus group. It is a flexuous rod containing a single-stranded, positive-sense RNA. Previous studies have shown that during infection Potato Virus X synthesizes subgenomic RNAs to express internal cistrons. These studies demonstrated that six subgenomic RNAs could be detected in total RNA extracts from PVX-infected tissue; however, only four internal ORFs are predicted within PVX genomic RNA. Furthermore, two particle size populations were detected, one containing genomic RNA and one containing the coat protein mRNA. In the present investigation experiments were designed to examine PVX-encoded subgenomic RNAs with regard to their total number, their coding properties, and their possible encapsidation.

Electron microscopic examination of crude extracts and partially purified virus preparations of PVX revealed that the virus particles exist in seven distinct size classes using a published virus isolation procedure developed for PVX. However, only a single RNA species was extracted from these partially purified PVX particles. For analysis of the RNA species encapsidated in the smaller particles, virus purification and RNA extraction procedures were developed to allow reproducible isolation of the smaller RNA species. Using these procedures, seven RNA species (6.4 kb, 3.6 kb, 3.0 kb, 2.1 kb, 1.8, 1.4 kb, and 0.9 kb) were detected from purified virus preparations by agarose gel electrophoresis under either nondenaturing or denaturing

conditions. Northern blot analysis using two virus-specific probes containing either 3' or 5' terminal genomic sequences indicated that the smaller RNA species were 3' coterminal but shared no homology with the 5' end. PVX viral RNAs were selected from an oligo dT column indicating the presence of a poly A tail. Translation of PVX viral RNAs in wheat germ extracts resulted in the synthesis of at least four virus-specific proteins with relative masses corresponding to ORFs encoding polypeptides of sizes 24,547 (ORF 2), 12,589 (ORF 3), 7,943 (ORF 4), and 25,080 (ORF 5). These proteins were probably expressed from the 1.4 kb, 1.8 kb, 2.1 kb, and 0.9 kb RNAs respectively. In rabbit reticulocyte lysate, one virus-specific protein from full-length PVX RNA was predominant corresponding in size to ORF 1 encoding a polypeptide of molecular weight 165,588. Three additional proteins were also detected corresponding in size to polypeptides predicted for ORFs 2, 4, and 5. Western blot analysis of proteins extracted from infected tissue using antisera prepared to virus-specific peptides indicated that at least five viral proteins are produced *in vivo*.

CHAPTER I. INTRODUCTION

The Potexvirus group is a small virus group, containing 11 members and 19 tentative members (1). Despite its small size, this group is of economic importance (2). Its members are responsible for reducing yields of important food crops such as potato, papaya, and cassava. The group members studied most intensively include papaya mosaic virus (PMV), narcissus mosaic virus (NMV), white clover mosaic virus (WCIMV), clover yellow mosaic virus (CIYMV), and potato virus X (PVX) (3, 4, 5, 6, 7,1). Potexviruses are defined as flexible, rod-like particles 470 to 580 nm in length and 13 nm in diameter (1). They reach high titers in systemically infected hosts. This group causes mosaic or ringspot symptoms on a wide range of monocotyledonous or dicotyledonous plants, but the host range of individual members is limited. Most members are transmitted by sap inoculation but not through seed or by insects (8). However, PVX is reported to be transmitted on the mouthparts of the grasshoppers *Melanoplus differentialis* (9) and *Tettigonia viridissima* (10). Fungal transmission of PVX by *Synchytrium endobioticum* has also been reported (11). WCIMV has been reported to be transmitted by aphids (12). Potato acuba mosaic virus is transmitted by the aphid *Myxus persicae* with the help of potato viruses A and Y (13). Most of the host plants are vegetatively propagated aiding in wide dissemination of virus members (1). It is possible that the viruses may overwinter in weeds making eradication difficult.

Chemical, Physical and Structural Properties

Potexviruses sediment as a single component in the analytical centrifuge and have sedimentation coefficients of 114 to 130 S. Other virus groups with shorter rigid (tobamoviruses) and longer flexible (potyviruses and carlaviruses) rod-like particle morphologies than the potexviruses have higher sedimentation coefficients of 190 S and 130 to 170 S respectively (14).

CD spectral analysis indicated that the proportions of RNA and protein by particle weight are 5% and 95%, respectively in the potexvirus members cymbidium mosaic virus (CybMV), WClMV, cassava common mosaic virus (CCMV), PVX, PMV, and potato acuba mosaic virus (PoAMV) (14). PVX RNA is composed of the base composition (molar percentages): G 22; C 24; A 32; and U 22. Similar values were reported for the RNAs of WClMV, PMV, CybMV, and PoAMV. Adenine is the most common base in all cases (14).

The molecular weight of capsid proteins of the potexviruses was determined by gel chromatography with SDS or guanidine hydrochloride, SDS gel electrophoresis, and/or amino acid analysis by peptide mapping (14). PVX coat proteins ranged from 22 kDa to 30 kDa. Koenig (15) reported that intact PVX coat protein migrates anomalously in SDS polyacrylamide gels resulting in high values. He also noted that PVX coat protein was susceptible to proteolysis during virus purification or in storage (16, 17, 18). Degradation was found to occur at the amino and carboxyl termini (19). The correct mass was estimated to be 26 kDa (17). For other potexviruses, the correct mass of the coat protein was estimated to

be 20 kDa (15, 16, 18, 20, 21, 22). Multiple bands were detected in SDS polyacrylamide gels. This result suggested that other potexvirus members were susceptible to plant proteases during virus purification as well (14).

Proteins of PMV and NMV reassembled into rod-like structures in a helical array in the absence of their respective genomes which indicated that the structural stability of the virus particle is determined by protein-protein interaction (14). In intact particles, the number of protein subunits per helical turn ranged from 6.8 for NMV to 10 for PVX to 11 for WCIMV. Protein subunits formed a helix with a pitch of 3.3 to 3.7 nm. The interactions between successive helix turns of PVX and NMV are weaker than those of TMV. This weakness may contribute to the flexibility of the potexvirus particle (14).

Circular dichroism (CD) and fluorescence emission studies of PVX intact particles and dissociated protein subunits were compared (23). PVX protein subunits reassembled into infectious particles only in the presence of genomic RNA. This result suggested that PVX particle stability was determined by ionic interactions between genomic RNA and protein subunits. There was no dramatic shift in the tertiary structure of the protein subunit when it became part of the intact virus. The CD spectrum of coat protein subunits predicted a conformation consisting of 40 % alpha helix, 5 % beta sheet, and 55 % random coil. CD analysis was done in the aromatic wavelength range since the coat protein contains 2 tyrosines, 12 phenylalanines, and 6 tryptophans. CD and fluoresence studies suggested that the tryptophans were in different environments in the intact virus and the dissociated subunits. This was

concluded to be the result of local subunit conformational changes or changes in subunit interaction involving tryptophan in the intact virus (23). CD analysis of the viral RNA alone indicated that 69 % of the genomic sequence is base-paired (23).

Effect of Potexviruses on Host Cells

Potexvirus-infected cells show nonspecific alterations: (1) the accumulation of starch, and (2) the disruption of chloroplast and cellular membranes (23). Potexviruses induce inclusions that occur in most plant tissues except the tracheid and sieve tubes (24, 25, 26, 27, 28). Often the virus-induced inclusions are most prominent in epidermal cells and most light microscope studies occur at this site. The most dramatic structural alterations within cells are inclusion bodies which are virus particle aggregates. They can be in concentrations high enough to fill the cell lumen. The pattern of inclusions that consist of aggregates of virus particles can be orderely (WCIMV, PMV) or random (PVX, ClYMV, CybMV) (14). The virus aggregates usually occur in the cytoplasm and are not membrane-bound. It is uncertain whether a host influences the shapes of these inclusion bodies. The formation of virus aggregates of PVX has been followed throughout infection in protoplasts on the electron microscope level (29). The first virus particles were scattered in the cytoplasm. Characteristic aggregates appeared late in infection.

In some cases, inclusion bodies have distinct shapes characteristic of a particular virus. Laminate inclusions (30) consisting of proteinaceous sheets with a beaded appearance are found in the cytoplasm and are unique to PVX infections. These sheets are sometimes arranged to form scrolls.

These inclusions are antigenically unrelated to PVX coat protein and are not involved in virus synthesis since they do not appear until after virus particles are present late in infection (30). ClYMV infections are associated with spindle-shaped inclusions of virus particles which are located adjacent to the nucleolus of the nucleus suggesting that replication may occur at this location (11). NMV, PMV, and cactus virus X (CaVX) infections are also associated with nuclear inclusions (11, 31, 32, 33)

Genome Organization

The potexvirus genome consists of a single-stranded positive sense RNA which is capped at the 5' terminus and polyadenylated at the 3' terminus (1). The genomic RNAs of five potexviruses have been sequenced (34, 35, 36, 37, 38) and found to share the following characteristics:

The potexvirus genome contains five to seven open reading frames (ORFs) (34, 35, 36, 37, 38). Five ORFs span the genome in succession while two ORFs occur internally within ORF 1 and/or ORF 5. ORF 1 comprises two-thirds of the genome encoding 147 to 186 kDa proteins. ORFs 2, 3, 4, and 5 occupy similar positions at the 3' terminal one-third of the genome encoding 24 to 26 kDa, 12 to 14 kDa, 7 to 11 kDa and 20 to 25 kDa proteins respectively (figure 1). A conserved pentamer, GAAAA, occupies the 5' most position behind the cap structure (38). The leader region is 80 to 100 nucleotides in length, rich in A and C nucleotides, and can be folded into a stable secondary structure (38). There are intercistronic regions of variable length between ORFs 1 and 2, and ORFs 4 and 5. ORF 3 overlaps ORFs 2 and 4. There is a conserved sequence, A C G G

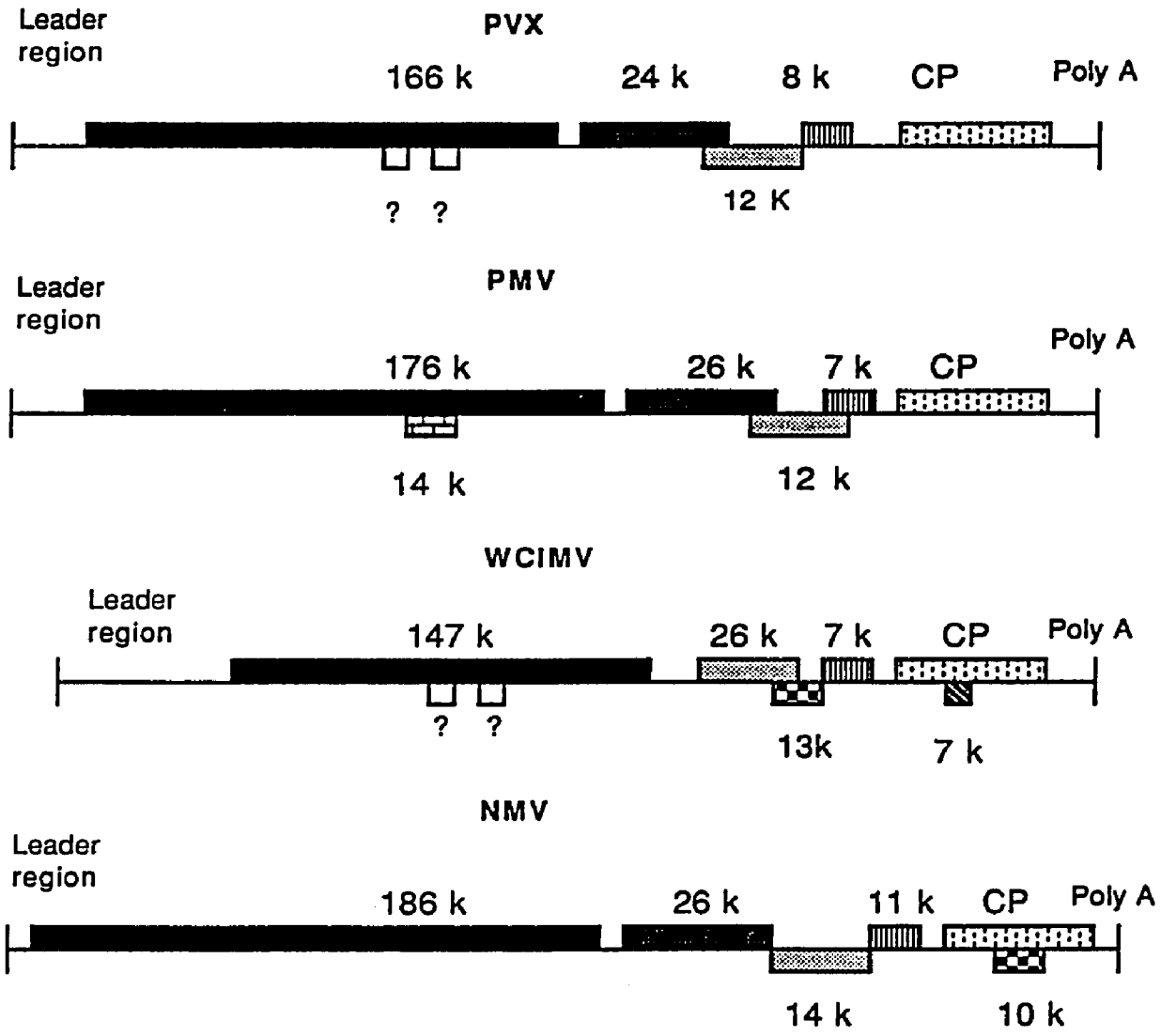


Figure 1. Schematic representation of the genomic organization of potato virus X and its comparison with other potex-viruses, papaya mosaic virus, white clover mosaic virus, and narcissus mosaic virus.

U U A A G U U U C C A U, upstream of the coat protein initiator codons and preceding PVX and NMV 26 k proteins (38). The two adjacent G residues in this conserved sequence are reported to be the cap site for the PVX coat protein mRNA (38). There are similar homologous sequences upstream of tobravirus cistrons (38). The coat protein occupies the 3' most coding region and is followed by an untranslated region of variable length. A consensus sequence, AAUAAA, is present in the 3' untranslated region and it precedes a poly A tail 75 to 300 nucleotides in length (38).

NMV, WCIMV, PMV, and PVX share conserved blocks of the putative replicase protein (ORF 1) at the N termini (400 amino acids) and C termini (800 amino acids). Similarities at the N termini range from 41 to 47 %. Similarities at the C termini range from 51 to 56 %. Separated functional replicase domains are present in hordeiviruses and tricornaviruses as well and are derived from RNAs 1 and 2 (38). A corollary to this motif is present in tobamo- and tobraviruses which have two replicase proteins separated by translational readthrough (38). An RNA-dependent RNA polymerase consensus sequence, GDD, is present at the carboxyl terminal domain of ORF 1 as well as a proposed NTPase-helicase (38). A conserved sequence, G X X G X G R (S T), is present in the ORF 1 products of NMV, WCIMV, PMV, and PVX. Immediately following this domain at the N terminal region of ORF 2 is another consensus sequence, G X G S T, which is present in the 24/26 kDa proteins of the potexviruses (38). There is 25 to 34 % homology among these protein products. The ORF 3 products, 12 to 14 kDa, share 43 % homology. There is limited sequence homology between the potexvirus protein products of ORF 4, PMV 7 kDa, NMV 11 kDa, PVX 8 kDa, and WCIMV 7 kDa. The capsid proteins (ORF 5) of PMV, PVX, WCIMV and NMV are 46 to 55 % homologous. There is no sequence homology between

the predicted protein products from PMV - ORF 1 and - ORF 2 (15 kDa), WCIMV -ORF 2 (20 kDa), and PVX - ORF 1 (12 kDa). Potexviruses also share significant homologies in the 5' noncoding region as well as the coding regions. The first 500 nucleotides of the 5' terminus of PMV RNA share 54.4 %, 50 %, and 49 % identity with the same regions in PVX, WCIMV, and NMV.

Interviral comparisons of amino acid sequences from different potexvirus RNAs with other plant viruses suggest that potexviruses are similar to tobamoviruses, potyviruses, furoviruses, and hordeiviruses (35). Comparison of sequences of potexvirus RNAs with those of other viruses allows the assignment of functions within the viral replication cycle (35). Homology exists between the 147 to 186 kDa proteins (ORF 1) of potexviruses with TMV 126/183 kDa and beet necrotic yellow vein virus (BNVV) 237 kDa proteins (34). The carboxyl terminal portion of the TMV 183 kDa protein is homologous to the carboxyl termini of the 147 to 186 kDa proteins of the potexviruses. The TMV carboxyl terminal domain is functionally equivalent to the viral replicase suggesting that the 147 to 186 kDa proteins of potexviruses are also (34). The 24 to 26 kDa proteins (ORF 2) share homology with the potyviral cylindrical inclusion proteins (34). These inclusion proteins are found in association with plasmodesmata early in infection suggesting a role in cell-to-cell movement. The 12 to 14 kDa proteins of the potexviruses (ORF 3) have hydrophobic domains characteristic of membrane-bound proteins. They share homology with BSMV and BNYVV proteins whose functions are still unknown. The 25 kDa protein from BNYVV RNA 3 shares homology with the potexvirus 7 to 11 kDa proteins (34). These proteins may function in vector transmission (34). The capsid proteins of potexviruses (ORF 5)

share sequence homology with those of several potyviruses (34). The significance of this homology is unknown at this time.

Despite the many similarities shared among the potexviruses, there are several striking differences:

(1) One obvious difference is the size of the potexvirus genomes which range in size from 5845 to 7015 nucleotides. The extra length is attributable to a size increase of the replicase protein (37).

(2) Although a consensus sequence was found upstream of the potexvirus coat protein gene (ORF 5), there are no consensus sequences upstream of internal ORFs 2, 3, and 4 within the potexvirus genome (37). Nonhomologous promoter sequences may be related to differential expression of these subgenomic RNAs. However, similar sequences upstream of ORFs 2 to 5 in PMV RNA have been reported suggesting that the subgenomic RNAs have similar 5' termini or share a similar mechanism for their synthesis (37).

(3) The number and position of the 3' consensus sequence, A A U A A A, is variable among the different potexvirus genomes. NMV contains two overlapping boxes immediately preceding the poly A tail (37). In WCIMV this sequence is present 13 nucleotides upstream of its poly A tail (37). In PVX and PMV, the terminal A A A residues of the consensus sequence are part of the poly A tail (37).

(4) An ORF is located within the coat protein cistron in similar positions

in the genomes of WCIMV and NMV encoding proteins of 10 kDa and 7 kDa respectively. This internal cistron is absent in PVX, PMV, and CIYMV. The small proteins encoded by this ORF share two blocks of sequence homology with their respective coat proteins (37).

(5) Computer analysis of PMV, WCIMV, and PVX negative sense genomes predict one or more ORFs (37). PMV contains two ORFs, -ORF 1 and -ORF2, encoding 15 kDa proteins. WCIMV and PVX contain a single ORF, -ORF 1, encoding a 20 kDa and 12 kDa protein respectively (37). These proteins have not been detected *in vivo* or *in vitro.*

The *Origin* Site of Assembly of Potexviruses

PVX was the first potexvirus to be reconstituted *in vitro* (39). Reconstitution was achieved at low (0.01 M) to moderate (0.1 M) ionic conditions, 22 degrees centigrade, and pH 6.0 to 6.2 (39). The method of PVX protein and RNA preparation affected reconstitution results. PVX RNA and protein prepared by freezing and thawing in 2 M LiCl were reconstituted successfully. Optical diffraction studies documented that reconstituted and native particles were identical (39). Reconstitution experiments were unsuccessful when protein subunits were prepared by calcium chloride, sodium dodecyl sulfate, or pyridine (39).

Reconstitution was successful only when genomic RNA was present which suggested that electrostatic interactions between genomic RNA and protein subunits were important in PVX assembly. Later studies, however, suggested a role of hydrophobic interactions were important in the assembly as well. A fluroescent dye, 8-anilino-1-napthalene sulfonate

(ANS), bound to hydrophobic domains of PVX protein subunits (40). Dye fluroescence increased when these tagged protein subunits and genomic RNA were reconstituted into intact particles which indicated that hydrophobic interactions were also important in virus particle stability. The *ori* site was not determined in these studies.

Papaya mosaic virus was the first potexvirus in which the *ori* site was identified (41). Reconstitution of PMV protein subunits into a helical array required low ionic concentrations, a pH range of 8.0 to 8.5, and a temperature range of 20 to 25 degrees centigrade (42). The assembly process was biphasic, initiation phase (temperature independent) and elongation phase (20 to 25 degrees centigrade) (42). Bancroft and Mackie (43) reported that *in vitro* assembly of PMV purified protein and RNA was a specific nucleation event in which the coat protein initiated assembly at the 5' terminus of genomic RNA. The 5' RNA initiation site occurred within the first forty nucleotides. This region was adenine rich and contained eight adjacent pentanucleotide sequences, G C A A A. Computer analysis supported this assertion when the secondary structure of the internal *ori* site in TMV RNA and the 5' end of PMV were compared. Both contained a hairpin nucleation structure (41). However, more recent studies by Erickson and Bancroft (42) found assembly initiation to occur internally in a random fashion at a pH range of 6.0 to 7.5, a low temperature, and no or low salt conditions. The assembly nucleation process under these conditions occurred within seconds. The reconstituted particles, however, had a segmented appearance which may have resulted from the helical units being out of phase. The overall affinity of protein for genomic RNA was higher at pH 6.0. At pH 4.0, long protein helices were formed in the absence of sodium chloride. The inhibitory effects of sodium chloride

at all pHs may affect the RNA secondary structure conformation. Less RNA confomation would occur at low temperature and no salt (42).

The 5' *ori* site was questioned when the coat protein mRNA was found to be encapsidated (43). This observation was reconciled by the following two explanations: (1) The 5' end can be folded into a secondary structure capable of initiating encapsidation (43), or (2) There is a secondary *ori* site within genomic RNA (43).

CIYMV assembly was also studied. CIYMV shared some assembly properties with PMV, but its assembly occurred at a lower pH (7) and subassembly polymers were detected (44). CIYMV purified proteins made two tubular structures without genomic RNA. Stacked ring structures with deviations were made at pH 8.0 and flattened helical structures were assembled at pH 5.0 to 5.5 (44). The *ori* site was not defined in these studies.

Recent studies of NMV have compared the 5' terminus of the genomic RNA and its coat protein mRNA. There is no direct sequence or structural homology at the 5' terminus. However, both termini have some secondary structure. It is suggested that the *ori* nucleation site in NMV may be present near the genomic 3' terminus within the coat protein coding region as is the case with the cowpea strain of TMV (38).

Gene Expression

Most of the early research concerning the potexviruses involved the

study of PVX. The research centered around the particle structure, determination of the infectious unit, and purification methods (45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55). PVX, as well as other potexviruses, is unique in that upon purification it forms insoluble, entwined, rope-like structures with components of cellular extracts adhering to its surface (49). Once satisfactory purification methods involving charcoal clarification (50) or chromatographic isolation (51) were developed for PVX, the coding properties of the genomic RNA were examined in *in vitro* translation sytems. *In vitro* translation of PVX genomic RNA in wheat germ extracts yielded a single 110 kDa protein (56). In a later study, two high molecular weight proteins, 180 and 145 kDa, were detected in rabbit reticulocyte lysate and wheat germ extracts (57). However, these proteins did not account for the total coding capacity of PVX genomic RNA. The protein products from the genomic RNA of WCIMV, as well as other potexvirus members, produced from *in vitro* translation systems were examined as well. Similar results were obtained. Two high molecular weight proteins were detected. Hybrid arrest translation studies indicated that the products were derived from the 5' end of the genome (58, 59, 60, 61, 62). However, *in vitro* translation results using PMV genomic RNA were unique in that the coat protein was detected as well. From these results, it was concluded that PMV RNA was cleaved in rabbit reticulocyte lysate to activate it for coat protein synthesis (61).

Potexvirus research advanced further when the particle morphology of the potexviruses was re-examined. PVX was the first potexvirus reported to contain two virus particle populations, one containing genomic RNA and one containing the coat protein mRNA (63). Previously, except for PMV, the coat protein of potexviruses was expressed *in vitro* from clones made to

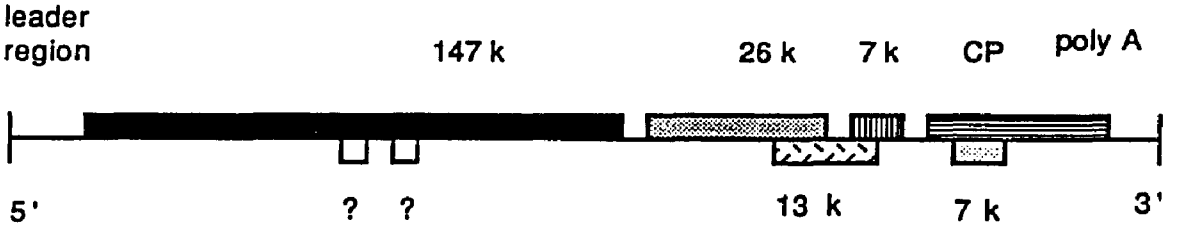
the 3' terminal region of the potexvirus genomes (58, 61 62, 63). After this discovery, two particle populations were detected in other potexviruses (60, 62, 64, 65, 66). Although two particle length populations were studied, histograms of PVX, WCIMV, daphne virus X (DVX), NMV, and PMV showed five to seven different particles sizes (54, 64, 65, 66, 67). The intermediate virus particle sizes were probably not examined because at the time of isolation from infected leaves (two weeks post inoculation), their presence was barely detectable.

The next important discovery concerning replication of the potexviruses occurred in 1987. Dolja, Grama, Morozov, and Atabekov (68) detected six subgenomic PVX RNAs in total RNA extracts from infected leaf tissue by Northern blot analysis. Two major RNA species, 2.1 kb and 0.9 kb, and four minor species, 3.6 kb, 3.0 kb, 1.8 kb and 1.4 kb were detected. (68). All subgenomic RNAs shared a 3' coterminal sequence with genomic RNA. In addition, double-stranded analogs of the single-stranded species were isolated (68). The replicative forms (RF) of full-length genomic RNA and subgenomic RNAs of PVX were examined (68). The 3' end of the complementary strand of double-stranded genomic RNA contained an unpaired G residue that was not present in the complementary strands of subgenomic RNAs. This observation indirectly suggested that complementary subgenomic RNAs cannot serve as templates for messenger subgenomic RNAs. An unpaired G residue at the 3' terminus of complementary RF strands has been found in two other plant virus groups, the hordeiviruses (69) and the cucumoviruses (70). After this discovery, other potexviruses were examined and multiple subgenomic RNAs were detected in polyribosomal and total RNA extracts by Northern blot analysis (60, 63, 65, 66).

Upon the detection of subgenomic RNAs in total RNA extracts, the replication strategy of the potexviruses became clear. The genomic RNA uses subgenomic RNAs to express internal cistrons. However, successful attempts to detect viral proteins from subgenomic RNAs in total RNA extracts and polyribosomal extracts used as templates in *in vitro* translation systems have not appeared in print.

To determine the coding capacity of individual potexviruses, research efforts turned to cloning and sequencing the entire genome. The first potexvirus to be cloned and sequenced was WCIMV (34). The sequence of WCIMV contained 5848 nucleotides and a poly A tail of 300 nucleotides. Computer analysis predicted five ORFs. A cap structure occupies the 5' most end of the genomic RNA. Immediately following the cap is a 108 nucleotide untranslated region. The first and second ORFs encode 147 kDa and 26 kDa proteins. There are two in frame amber codons within ORF 1 encoding 150 kDa proteins. No evidence has confirmed the existence of these two readthrough proteins *in vivo* or *in vitro.* The third ORF overlaps ORFs 2 and 4 ORFs 3 and 4 encode 12 kDa and 7 kDa proteins respectively. ORF 5 encodes the coat protein, a 20 kDa protein. The coat protein occupies the 3' most coding region followed by an untranslated region and a poly A tail. Two additional ORFs have been identified. The first ORF, encoding a 10 kDa protein, is contained completely within ORF 1. The second ORF, a 20 kDa protein, is encoded on the negative sense genomic RNA (figure 2).

Recently the nucleotide sequence of PVX RNA was determined (36). Computer analysis of PVX genomic RNA predict five ORFs. The 5' end has an 84 nucleotide leader sequence and the 3' end contains an untranslated



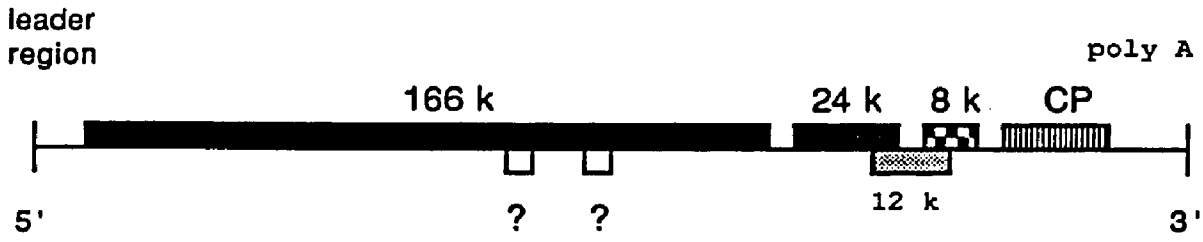
Genomic RNA region	Bases	Proteins
Leader region	1 - 107	None
ORF 1	108 - 4104	147 k
ORF 2	3995 - 4709	26 k
ORF 3	4683 - 5030	13 k
ORF 4	4960 - 5154	7 k
ORF 5	5166 - 5738	CP
Noncoding region	5739 - 5849	None
Poly A tract	5850 - ?	None

Figure 2. The arrangement of open reading frames of white clover mosaic virus.

region of 76 nucleotides plus a poly A tail. The first and second ORFs encode 165,588 and 24,622 Da proteins, respectively. The third ORF begins within the 3' terminal region of ORF 2 and extends beyond the 5' terminal region of ORF 4. It encodes a 12,324 Da protein. ORF 4 encodes a 7,595 Da protein. The fifth ORF encodes the coat protein, a 25,080 Da protein (figure 3).

The genomic organization of WCIMV and PVX are very similar. Computer analysis predicts five ORFs. ORFs one through five appear in succession along the genome with three overlapping ORFs encoding proteins of similar sizes. WCIMV contains three additional ORFs, two within ORF 1 and one on the negative sense strand (34, 36). Literature reports of PVX containing six subgenomic RNAs suggest that at least two additional unidentified ORFs are also present. Their probable location is within ORF 1 based upon the sizes of the two additional RNAs reported, 3.6 kb and 3.0 kb.

Unable to detect proteins encoded within ORF 2, ORF 3, and ORF 4 from viral RNAs, researchers resorted to experimental approaches in which truncated clones spanning the entire genome were used to produce synthetic RNA transcripts by *in vitro* transcription. *In vitro* translation of the transcripts resulted in the expression of viral proteins indirectly (71, 72). Clones spanning genomic PVX RNA were examined (71). Synthetic RNA transcripts of full-length cDNA, pBS118 and two 5' terminal truncated clones of the 165 kDa protein, clones p26 and p11, efficiently expressed the 165 kDa protein and its truncated derivatives, a 35 kDa protein and a 140 kDa protein in rabbit reticulocyte lysate. It is important to note that numerous minor bands were detected upon the translation of genomic PVX RNA and synthetic transcripts from p11. The presence of a cap structure



Genomic RNA region	Bases	Proteins
Leader region	1 - 84	None
ORF 1	85 - 4453	166 k
ORF 2	4486 - 5164	25 k
ORF 3	5147 - 5492	12 k
ORF 4	5427 - 5637	8 k
ORF 5	5650 - 6361	25 k
Noncoding region	6362 - 6438	None
Poly A tract	6439 - ?	None

Figure 3. The arrangement of the open reading frames of potato virus X genome.

was not necessary for the expression of the viral synthetic RNA transcripts, but it did have a significant stimulatory effect on the level of translation.

Synthetic RNA transcripts of clone pA103, encoding the coat protein were expressed in Krebbs 2 extracts and confirmed by immune precipitation. To determine if overlapping ORFs 3 and 4 were expressed, three clones, two containing ORFs 3 and 4 of different lengths since the 5' termini of ORF 3 is unknown, and one containing ORF 4, were prepared. Two clones, pM69 and p142, were dicistronic encoding 8 kDa and 30 kDa proteins as well as 5 nucleotides and 177 nucleotides upstream of the 8 kDa protein. Synthetic transcripts were translated in Krebbs 2 extracts and two products were translated, the 8 kDa and the 30 kDa. The 8 kDa protein was more efficiently expressed in p142 containing the 177 nucleotide leader sequence. Expression of the coat protein was not expected on the dicistronic message since its cistron was located internally. However, mechanisms of internal initiation, leaky scanning and re-entry of ribosomes have been reported recently in eukaryotic dicistronic messages (73, 74, 75). Translation of synthetic transcripts of a monocistronic clone, pK71, yielded a 12 kDa protein.

Both the 8 kDa and 12 kDa proteins have predicted hydrophobic domains characteristic of membrane-bound proteins. Synthetic transcripts of p11, pK71, and pA103 were translated in membrane-enriched Krebbs 2 extracts. The translation products were separated on a sucrose gradient and the smooth membrane fraction was enriched with the 8 kDa and 12 kDa proteins, but not the coat protein, suggesting that these proteins are membrane-associated (71).

Truncated clones containing the 3' terminal one-third region of ClYMV were used to express viral proteins as well (72). Computer analysis of the genomic RNA of ClYMV predicts seven ORFs. The 5' most proximal ORF 1 encodes a 191 kDa protein. Overlapping ORFs 2, 3, and 4 appear in succession encoding 25 kDa, 13 kDa and 6.5 kDa proteins respectively. The 3' most proximal ORFs are 5 and 5a encoding 28 kDa and 27 kDa proteins (figure 4). Synthetic transcripts of truncated clones containing ORFs 4, 5, and 5a efficiently expressed proteins of predicted sizes 6.5 kDa, 23 kDa, and 30 kDa, *in vitro* in rabbit reticulocyte lysate. The 5a ORF occurs within ORF 5. The 5' ends of the two most abundant subgenomic RNAs, 2.1 kb and 1.0 kb, isolated from polyribosomal and total RNA extracts were mapped at the nucleotide level and contain a cap structure (72).

The most recent study of potexvirus replication resulted in the detection of two subgenomic RNAs from purified ClYMV particles by Northern blot analysis. This result suggests that the subgenomic RNAs produced during infection are encapsidated and that the origin site of assembly may not be at the 5' end (72). At the present time, there is still no direct evidence that the predicted ORFs 2, 3, and 4 within the genomic RNA of PVX or other potexviral genomic RNAs are functional.

Parallels with TMV

Although TMV (76) and PVX (2) are members of different virus groups, the tobamoviruses and potexviruses, respectively, their replication strategy is similar. Both viruses have a rod-like particle morphology. A single genomic RNA which encodes all replication functions is encapsidated (76, 2). During infection, both synthesize subgenomic RNAs to express internal cistrons (68, 77). However, more is known about TMV

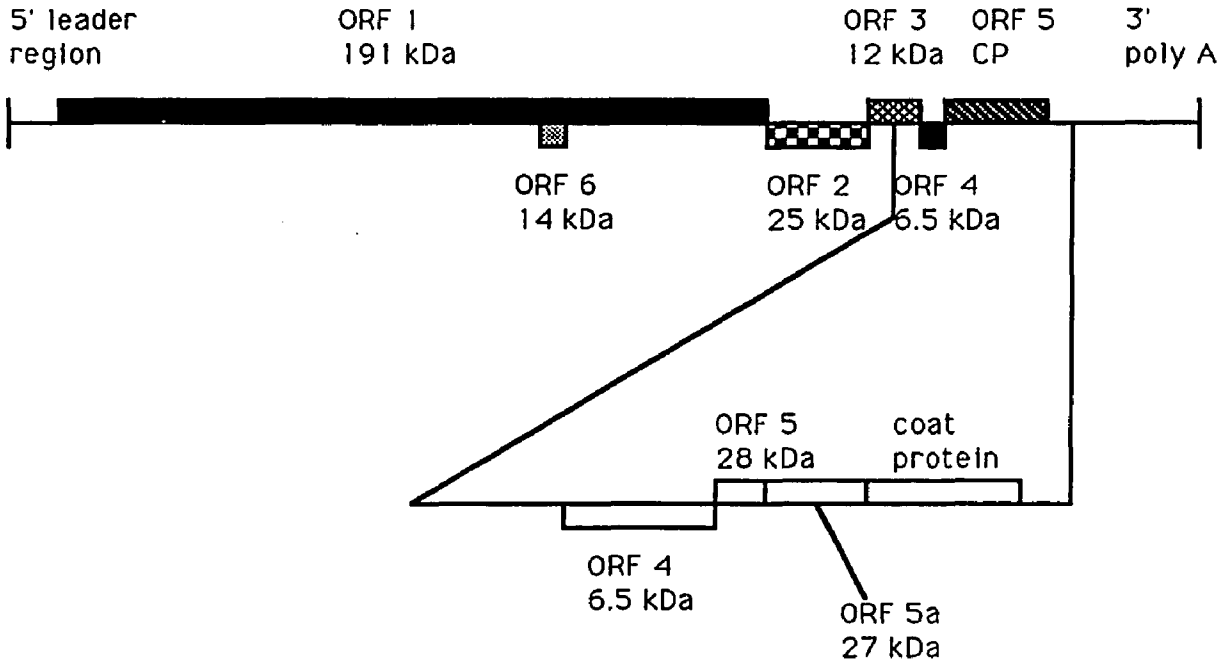


Figure 4. Schmatic representation of the genome organization of clover yellows mosaic virus.

replication (78), despite the fact that PVX and TMV were discovered about the same time (79, 80). Early studies (1900's to the 1940's) concerning TMV were done on the common strain. Structural parameters, purification methods, assembly studies, and *in vitro* translation of genomic RNA were the first aspects studied. TMV was initially defined as a single, full-length rod 300 nm in length (76). Different purification methods such as charcoal clarification (81), differential centrifugation (82), PEG (83), or DEAE chromatography (51) were developed to prevent aggregation. Once intact virus particles were obtained, the *origin* site of assembly was mapped at a location 5' of the coat protein gene (84). Early *in vitro* translation studies using TMV genomic RNA resulted in the synthesis of two high molecular weight proteins, 126 kDa and 183 kDa (85). Although TMV was defined as a single rod, electron micrographs of purified or crude extracts of TMV displayed less than full-length rods (86). These intermediate rods were dismissed as purification artifacts. In the early 1950's, however, Bawden, Pirie and others re-examined the less than full-length rods of TMV (87, 88). Experimental results (88) suggested that the smaller rods were not purification artifacts or *in vivo* breakage products within the plant cell. Still later in the 1970's, the shorter rods of three TMV strains, common, wheat, and cowpea were characterized (89). Two intermediate classes of particles, I_1 and I_2, encapsidated subgenomic RNAs. These RNAs were 3' coterminal with their genomic counterpart and upon translation, 30 kDa and 50 kDa products were detected. In 1974, two particle

populations of the cowpea strain of TMV were detected (90). One contained genomic RNA and the other contained the coat protein. *In vitro* translation products of TMV viral RNAs of the cowpea strain were examined (91). Three RNAs were extracted from purified virus preparations. The full-length RNA synthesized a 130 kDa protein. The intermediate and small rods encoded 30 kDa and 17 kDa proteins respectively. The *ori* site was mapped to a region within the coat protein gene (92). The most recent studies examining TMV replication suggest that four RNAs are synthesized during TMV infection and all RNAs except the coat protein mRNA are encapsidated (78). In the cowpea strain, the coat protein mRNA is encapsidated as well (91) Familiarity with TMV research with regard to the examination of the number, coding properties and possible encapsidation of its subgenomic RNAs should provide insight into approaches and interpretations applicable to PVX in the examination of its subgenomic RNAs from purified virus preparations.

PVX research has progressed slowly because purified virion preparations form insoluble rope-like structures (47). Despite this difficulty with PVX, the history of the determination of the replication strategy of PVX has parallels with TMV.

At the inception of PVX research, the same parameters as TMV were examined (ie. particle structure, purification methods,

assembly and *in vitro* translation studies). Like TMV, PVX was defined as a single, full-length rod (2). Purification methods were developed for PVX, but unlike TMV, a suitable purification method to prevent aggregation has still not been developed despite the fact that literature dealt with this aspect over ten years (84, 52, 53, 54, 55). PVX assembly studies were not as straightforward as TMV (12). The *ori* site is still undetermined. Indirect evidence from another potexvirus, PMV, suggests that the assembly site is at the 5' terminus of PVX genomic RNA (41, 42, 43, 44). Early *in vitro* translation studies with PVX genomic RNA yielded two high molecular weight proteins, 145 kDa and 180 kDa, like TMV (56, 57).

In this investigation, experimental results suggests that PVX replicates like the cowpea strain of TMV. Therefore, it is important to review the approaches taken in the delineation of the replication strategy of TMV in areas that have relevance with PVX.

Initially TMV was defined as a single rod (76). Sizes longer than 300 nm were regarded as end-to-end aggregation products, and sizes smaller than 300 nm were regarded as breakage products of purification (93). In later studies regarding the anatomy of TMV, controversy arose concerning the particle size of TMV (94). There was agreement that rods longer than 300 nm were artifacts. It was the origin of rods shorter than 300 nm that was in dispute. Chemists, biochemists, and physicists assumed that particles

of a particular virus had a constant width, length, composition and sequence of amino acids and nucleotides. Any deviation from a determined value was regarded as a contaminant and removed (88). Many concluded that TMV had a uniform length (95, 96, 97, 98, 99, 100, 101). In the same era, PVX was also defined as a flexible rod of uniform length (2).

Biologists, aware of variation, were open to the possiblity that an activity could be associated within a loosely defined range (88). They regarded the shorter rods as natural products of TMV replication (88, 102, 103, 104, 105, 106). The following experimental results (88) suggested that the shorter rods were not preparative artifacts: (1) The grinding of TMV-infected leaf tissue in a mortar with a pestle caused some rod breakage, but did not account for more than two to three percent of the shorter rod population. Grinding by a motor-driven homogenizer did not effect rod length. These findiings suggest that the shorter rods were not purification artifacts. (2) Gradient purified full-length TMV rods were examined by the electron microcope. Ninety-five percent of the particles were 300 nm and five percent were within 200 and 300 nm. These results suggested that the shorter rods were not generated during specimen preparation for electron microscopic examination. (3) To address the possibility of *in vivo* breakage of the 300 nm rods within the plant cell, TMV-infected leaves were inoculated with $C^{14}O_2$. The virus particles were separated according

to size by sucrose density gradient fractionation. The specific activity of different fractions are dependent upon the proportions of rods of different sizes which were present. If the shorter rods are breakage products of the virus originally synthesized as 300 nm rods, then the specific activities should be identical. However, the specific activity of the labeled full-length rods was different than the labeled shorter rods suggesting that the shorter rods are not *in vivo* breakage products of the 300 nm rods. (88). Shorter rods of PVX were depicted in electron micrographs during this same time period, the 1930's, but extended throughout the 1960's. No one considered the possiblity that the less than full-length rods of PVX contained subgenomic RNAs. They were simply regarded as purification artifacts (47, 48, 49, 54).

Throughout the study of TMV, there has been no consensus concerning the number of viral RNAs present during infection. Initially, ten single-stranded RNAs from total RNA extracts of PVX-infected leaf tissue were detected (107). Some of these RNAs were generated by the extraction method (107) and other RNAs were electrophoretic variants (107). Years later another lab reported the presence of seven subgenomic RNAs with double-stranded counterparts (108), and still another group reported thirteen double-stranded RNAs in total RNA extracts from TMV-infected leaf tissue (109). Most virologists agreed early on that the common strain of TMV contained one particle population, and more recently that the

cowpea strain of TMV contained two encapsidated viral RNAs associated with infection, a genomic RNA and a subgenomic mRNA encoding the coat protein. By the 1980's, it was agreed that PVX contained two particle populations as well. One particle population contained genomic RNA and the other contained the coat protein mRNA (63).

Subsequent studies reported that several TMV strains, common, wheat, and cowpea, contained multiple less than full-length rods in addition to the two particle lengths previously reported (89). RNA was extracted and discrete size classes were detected. A class of RNA, ranging from 0.68 to 1.7 x 10^6 in molecular weight among the different strains, was found to encode a 30 kDa protein. The rods containing this RNA were designated I_2 particles. In later studies with TMV, a group re-examined the number of single- and double-stranded virus-related mRNAs. Upon completion of reconstitution experiments, it was concluded that four viral RNAs were present during infection, genomic RNA, I_1 RNA, I_2 RNA, and LMC RNA. Four double-stranded counterparts were also detected (92). One class of RNA, previously unidentified, was found to encode a 50 kDa protein. The rods encapsidating this RNA were designated I particles. This is the current status on the number of viral RNAs present during TMV infection. The most recent literature on PVX regarding replication states that during infection PVX synthesizes seven RNAs (68). Two of these RNAs are encapsidated, the genomic RNA and the coat

protein mRNA (63). The coding properties of the five RNAs not encapsidated have not been examined directly. No one has reported particle lengths containing PVX subgenomic RNAs 3.6, 3.0, 2.1, 1.8, and 1.4 kb.

By convention, virus identification is determined by the selection of a class of particles responsible for infectivity. The shorter rods were not infectious. This practice may be responsible for many researchers dismissing the smaller sizes of TMV and PVX as purification artifacts.

Research Objectives

The number, coding properties, and possible encapsidation of PVX viral RNAs are still yet to be determined. In this dissertation research, the following experiments were performed to provide more information on these questions:

(1) PVX-infected crude leaf extracts and purified virus preparations were prepared for SSEM and quick dip preparative analysis for examination in the electron microscope.

(2) Published PVX virus purification and RNA extraction procedures did not allow for the detection of smaller RNAs from purified virus preparations, so new virus purification and RNA extraction methods were developed for reproducible isolation of

these smaller RNAs.

(3) The number and size of purified virus particles and their RNAs were examined at time intervals of 5, 7, 9, 11, and 13 days post inoculation.

(4) Northern blot analysis experiments using PVX DNA fragments containing the 5' and 3' sequences of genomic RNA were performed to determine if the smaller RNAs isolated from purified virus preparations were fragments or encapsidated subgenomic RNAs.

(5) PVX viral RNAs were prepared for an oligo dT column to determine if they contained a poly A tail. Subgenomic RNAs typically have a poly A tail if it is present on the genomic RNA.

(6) PVX viral RNAs were translated in wheat germ extracts and rabbit reticulocyte lysate to determine their messenger activity.

(7) Primer extension experiments were designed to map the 5' ends of the smaller RNAs isolated from purified virus preparations.

(8) Antisera was prepared to peptides containing sequences present in ORFs 1, 2, 3, and 4 to detect PVX-specific proteins synthesized *in vivo* during infection.

CHAPTER II. MATERIALS AND METHODS

PVX strains and hosts.

A PVX strain (ATCC) was purchased from American Type Culture Collection. The PVX strains, C, G, and HB, were obtained from the Center for International Potato Research. PVX strains, Robert's and V349, were received from Dr. Purcifull.

PVX propagation hosts included: *Nicotiana glutinosa, Nicotiana tabacum var. Samsun, Nicotiana tabacum var. Xanthi, Nicotiana tabacum var. NC95, Nicotiana tabacum var. H425, Datura stramonium, Rutgers* tomato, and *Gomphrena globosa.* WClMV propagation hosts included: *Pisum sativa,* and *Datura stramonium.* NMV propagation hosts included: *Nicotiana benthamianum* and *Datura stramonium.* PMV was amplified in papaya and *Nicotiana benthamianum.*

Serological techniques

1. Serologically Specific Electron Microscopy (SSEM)

SSEM is a serological technique designed for the electron microscope to identify and study the morphology of plant viruses (110). A carbon-coated formvar film on a grid was used as a substrate for attachment of a specific antiserum prepared to the capsid protein of the virus examined. The grid was placed on a drop of antiserum for 30 minutes. It was then rinsed by being placed on drops of buffer and finally on a drop of a purified virus preparation or an infected leaf extract. After one hour, the grid was again placed on drops of buffer and washed in a steady stream of water to remove nonspecifically bound material, stained with uranyl acetate, and examined with an electron microscope. Two negative controls were included in each experiment: (1) capsid protein antisera was incubated on healthy leaf extracts, and (2) capsid protein antisera prepared to a virus member of

different group was used as substrate and subsequently incubated on purified virus preparations of the potexvirus examined.

2. Decoration

Decoration is a serological technique designed for the electron microscope to determine serological relatedness among similar plant viruses (111). Immuno-decoration is the reverse procedure of SSEM. A carbon-coated, formvar filmed copper grid was placed on a drop of virus preparation. After incubation, the grid was placed on a drop of antiserum. It was then placed on a drop of uranyl acetate and examined with the electron microscope. A beaded appearance of the virus particles indicates cross-reactivity of the antiserum. Since tobacco mosaic virus (TMV) is a common contaminant in solanaceous hosts due to its high infectivity and stability, antiserum to TMV was used as a negative control.

Measurement of virus particle lengths

A specimen and calibration standard (carbon replica of a waffle diffraction grating) were photographed during the same examination period at the same magnifcation. A negative of the standard was used to produce a specimen photograph of a predetermined magnifcation. The following formula was used to calculate the virus particle lengths (112):

$$\text{Actual particle size} = \frac{\text{size of particle in micrograph} \times 10^6}{\text{final micrograph magnification}}$$

The length of the virus particle was measured in millimeters on a photograph by a ruler. The size of the particle was multiplied by a conversion factor (10^6) to determine the actual particle size in nanometers. The final micrograph magnification was determined by multiplying the instrument magnification at which the specimen was photographed by the negative enlargement magnification.

Quick dip virus specimen preparation for electron microscopic examination

A formvar filmed, carbon-coated copper grid was placed on a sample drop (crude extract or purified virus preparation). Components in the sample drop were allowed to attach nonspecifically to the filmed grid for 5 to 10 minutes. The grid was removed from the drop and placed on a drop of 1 % aqueous uranyl acetate for 5 minutes. The grid was removed and air-dried (113).

Instrument specifications for specimen examination on the electron microscope: 80 kV, Objective lens #2, Condenser lens #2, magnification range of 5000 to 20,000X.

Virus purification methods

The three methods used most often throughout the early stages of this investigation include:

Method #1 (Acidification/$AgNO_3$ / PEG)
Leaf tissue was homogenized in 0.25 M borate buffer (pH 8.0) and 1% Na sulfite. The extract was acidified to pH 5.5 and centrifuged. The supernatant was recovered and silver nitrate was added to remove fatty acids. The mixture was incubated 3 hours at room temperature. The sample was centrifuged and the supernatant recovered. Then PEG was added to the supernatant and stirred at four degrees for sixteen hours. The virus was recovered in the pellet after centrifugation (55).

Method # 2 (butanol/Triton X/PEG)
A method as described in (114) was followed with the

following modifications:
Frozen infected leaf tissue (100 g), harvested two weeks post inoculation, was minced in a Waring blendor for two minutes at four degrees in 150 ml of 20 mM HEPES pH 7.5, 0.1 % Na sulfite, 1.0 % (v/v) ascorbic acid, and 8 % (v/v) butanol. The extract was filtered through cheesecloth and centrifuged at 5000 rpm for ten minutes. The supernatant was saved and mixed with Triton X 100 (to a final concentration of 1 %). Virus was recovered by the addition of 6% (v/v) PEG and 0.25 M NaCl with stirring at four degrees for four hours. The samples were centrifuged at 10,000 rpm for ten minutes at four degrees. The pellet was resuspended with a glass tissue homogenizer and recentrifuged at 8000 rpm for five minutes. The supernatant was saved and the virus was stored in one ml aliquots in sterile eppendorf tubes at - 20 degrees.

Method # 3 (charcoal)
The procedure, designed by McLean and Francki, was followed as described in (50).

Three other methods used once in preliminary studies comparing different virus purification methods include:

Method #4 (PEG)
The protocol described in (53) was followed.

Method # 5 (differential centrifugation)
The procedure in (52) was followed as described.

Method # 6 (DEAE chromatography)
The chromatographic procedure followed in (51) was designed for the purification of PVX and TMV.

The virus purification method developed during this investiga-

tion is included in Method # 7:

Method # 7

Fresh infected leaf tissue was deribbed and weighed. Extraction buffer (10 mM HEPES, pH 7.5, 1 mM ethylenediamine tetraacetic acid, 8% butanol, 0.1 % Na sulfite) was added in a ratio of 10:1 relative to mass of leaf tissue. The tissue was homogenized at four degrees centigrade at a high speed setting in a pre-cooled glass tissue homogenizer for two to three minutes. The crude extract was filtered through cheesecloth and centrifuged at 8000 rpm for ten minutes at four degrees centigrade. The supernatant was saved and Triton X 100 was added to a final concentration of 1%. The virus was recovered by the addition of polyethylene glycol and sodium chloride to a final concentration of 4% and 0.1 M respectively. The sample was stirred at four degrees centigrade for 1.5 hours. The virus sample was centrifuged at 8000 rpm for 15 minutes at four degrees centigrade. The pellet was saved and resuspended in 10 mM HEPES pH 7.5 with a glass tissue homogenizer. The sample was centrifuged at 5000 rpm for five minutes at four degrees centigrade. The supernatant which contained the virus particles was stored at -20 or -70 degrees centigrade in one ml aliquots.

RNA extraction methods

Method #1 (STE/Proteinase K)

The virus concentration was adjusted to 2 mg/ml and resuspended in 0.1 M NaCl, 1 mM EDTA, 0.05 M Tris-Cl pH 7.5, 3 % SDS and proteinase K at a concentration of 100 ug/ml (115). The virus mixture was incubated at 37 degrees for 30 minutes, extracted in phenol twice, and 2 volumes of ethanol were added to the aqueous phase. The virus preparation was stored at - 20 degrees centigrade overnight.

Method # 2 (SSC/SDS)

PVX virions were extracted by the addition of 1 X SSC and 3 % SDS to a virus concentration of 0.1 - 1.0 microgram/ml. The mixture was phenol extracted twice, and the aqueous phase treated with two volumes of ethanol. This is a modification of the procedure as described in (36).

The RNA extraction method developed during this investigation is as follows:

Method #3

The virus concentration was diluted to a concentration of 0.1 to 1.0 mg/ml. Sodium dodecyl sulfate and STE were added to final concentrations of 3 % and 2X respectively. Two volumes of phenol/chloroform were added. The sample was vortexed for two minutes and centrifuged at 8000 rpm for 10 minutes at four degrees centigrade. The aqueous phase was recovered and was vortexed for two minutes in one volume of phenol/chloroform. The sample was centrifuged at 8000 rpm for 10 minutes and the aqueous phase was recovered. Two volumes of ethanol were added and the sample was stored at -20 degrees overnight.

Northern blot analysis

1. Preparation of radiolabeled cDNA probes

Two cDNA fragments containing nucleotides 29 through 733 of PVX genomic RNA (5' terminal sequences) and nucleotides 5249 to 6436 of genomic RNA were random prime labeled using ^{32} P-dATP (10 mCi/ml) as described in (116). The specific activities of the two probes were 1.2 x 10^9 dpm/microgram.

2. Protocol for the transfer of viral RNAs to Zeta-probe membranes, hybridization conditions and washes

RNAs were separated on a 1.0 % agarose gel under denaturing

conditions (117). The RNAs were transferred onto a Zeta-probe membrane under alkaline conditions (50 mM sodium hydroxide) by capillary action. The filter was soaked in 20 X SSC, air-dried, and baked at 80 degrees centigrade for 2 hours under vacuum. The filter was pre-hybridized (2 hours) and hybridized (12 to 16 hours) at 62 degrees centigrade (prehybridization buffer and hybridization buffer: 1 mM EDTA, 40 mM sodium phosphate pH 7.2, 7% sodium dodecyl sulfate) and washed (two washes, thirty minutes each) at 55 degrees centigrade (wash #1: 1 mM EDTA, 40 mM sodium phosphate pH 7.2, 5% sodium dodecyl sulfate; wash #2: 1 mM EDTA, 40 mM sodium phosphate pH 7.2, 1% sodium dodecyl sulfate). The filter was blotted dry and exposed to XAR X-ray film.

Protocol used for the isolation of RNAs containing a poly A tail

A Fast Track mRNA isolation kit (Invitrogen) was used to isolate poly A + RNA from purified viral RNA and total RNA preparations. The procedure followed included:

Lysis buffer containing 0.02 volumes of proteinase was added per gram of plant tissue or virus weight. Oligo dT cellulose was pre-treated with 2 ml of binding buffer for one hour at room temperature. The hydrated column was spun down for 2 minutes at 5000 g. The cellulose pellet was resuspended in 0.5 ml of binding buffer until the RNA preparation was ready. One milliliter of purified virus was placed in a sterile 30 ml polypropylene tube. Sodium chloride was added at a final concentration of 0.5 M and the suspension was vortexed thoroughly. The sample was added to oligo dT in a sterile polystyrene tube. The tube was rocked slowly at room temperature for 60 minutes. The cellulose was recovered by centrifugation and resuspended in 20 ml binding buffer. This step was repeated three times. After the last wash, 0.4 ml of binding buffer was added. The cellulose was placed in a

conical tube and spun in a microcentrifuge for 10 seconds at 5,000 g. The column was removed and the liquid decanted. This step was repeated twice. RNA was recovered by adding 0.2 ml elution buffer to the cellulose. The sample was spun, the liquid was recovered. RNA was precipitated by adding 0.15 volumes of 2 M sodium acetate and 2 volumes of ethanol. The sample was incubated at - 70 degrees centigrade for 30 minutes. The tube was spun at 16,000 g for 15 minutes and the RNA pellet was resuspended in elution buffer.

Cell-free synthesis in wheat germ extracts

Wheat germ extracts were obtained from Promega. The conditions for translation using PVX viral RNAs as templates in mRNA-dependent wheat germ extracts have been described in (118). Optimized magnesium ion, potassium ion, and viral RNA concentration optima were determined: 1.0 mM magnesium, 90 to 110 mM potassium and 100 ug/ml PVX viral RNAs. Incorporation levels of ^{35} S-methionine into viral proteins were determined by assaying a reaction mixture volume of two microliters on Whatman 3 MM filter discs for acid-insoluble radioactivity.

Cell-free synthesis in rabbit reticulocyte lysate

Untreated rabbit reticulocyte lysate was obtained from Promega. Translation conditions for the expression of PVX viral RNAs in mRNA-dependent rabbit reticulocyte lysate have been described in (119). Optimized cocentrations of magnesium (0.5 mM), potassium (90 mM), and PVX viral RNA concentration (100 micrograms/ml) were added to the translation mix. The incorporation levels of ^{35} S-methionine into viral proteins were determined by assaying two microliters of the reaction mixture on Whatman 3 MM filter discs for acid-insoluble radioactivity.

Primer extension procedure

1. Chemical synthesis and purification of cDNA fragments

Sequences (15 to 18 bases in length) complementary to bases located about 20 bases downstream of ORFs 1, 2, 3, 4, and 5 were chosen from the published nucleotide sequence of PVX genomic RNA (strain X3) (36). Oligodeoxynucleotides were synthesized on an Applied Biosystems DNA synthesizer (Model 380 A). After termination of oligodeoxynucleotide synthesis, the preparation was incubated with one milliliter of concentrated ammonium hydroxide for 12 hours at 50 degrees centigrade to remove the blocking groups. The solution was dried to a powder using a rotary evaporator. The powder was resuspended in one milliliter of triethylammonium bicarbonate (TEAB) pH 7.6 and then evaporated. This TEAB step was repeated two more times. The final pellet was redissolved in three milliliters of TEAB and the fragments were recovered from a SEP-pak cartridge (120). This cartridge removed incomplete sequences from the completed chains. The liquid eluted from the cartridge was dried and the oligonucleotide fragments were dissolved in one milliliter of 80 % acetic acid and incubated at room temperature for 25 minutes to remove the 5' terminal blocking groups. The sample was dried, redissolved in water, and dried again. The deprotected product was purified further on a 20 % denaturing polyacrylamide gel and eluted as described in (120).

2. End labeling of primer

The labeling mixture contained 7.5 nanograms of primer, 30 microcuries of gamma ^{32}P-ATP (10 mCi/ml), 0.05 M Tris-Cl pH 7.5, 0.01 M magnesium chloride, 0.01 M dithiothreitol, and 4 units of polynucleotide kinase. The reaction mixture was incubated at 37 degrees centigrade for 30 minutes. The reaction was stopped by the addition of 1 ul of 0.5 M EDTA pH 7.5, heating samples at 100 degrees centigrade for five minutes and quenching on ice.

Unincorporated label was separated from incorporated on a Sephadex G-25 column (121). The specific activity of the probe was 5.0 x 10^9 dpm/microgram.

3. cDNA-RNA hybridization

Purified PVX viral RNAs (5 ug) and 2.5 ng of labeled primer were mixed together and dried. The pellet was redissolved in 1.5 microliters of water, incubated at 65 degrees centigrade for five minutes and quenched on ice.

4. Primer extension by reverse transcriptase and the analysis of cDNA products in a denaturing polyacrylamide gel

Primer extension of cDNA-RNA hybrids was performed as described in (122). Marker lanes were prepared from an M 13 DNA template using a Sequenase kit (United States Biochemicals). After primer extension, samples were denatured in boiling water for five minutes in an equal volume of 90 % formamide, 20 mM ethylenediamine tetraacetic acid, 0.1 % xylene cyanol, 0.1 % bromophenol blue. Three microliters of each sample were loaded and analyzed on a 10 % polyacrylamide-8.3 M urea gel.

Protocols used for dot blot hybridization analysis and western analysis

1. Preparation of virus-specific peptides

To determine the number of viral proteins produced *in vivo* during infection, peptide sequences of the 166 kDa (ORF 1), 24 kDa (ORF 2), 12 kDa (ORF 3), and 8 kDa (ORF 4) proteins were chosen from published amino acid sequences deduced from the nucleotide sequence of PVX genomic RNA (strain X3) (36). The location of the sequence on the protein was not important except for the 166 kDa protein. The sequence chosen in this case was limited to the carboxyl terminus in case the 3.6 kb and 3.0 kb subgenomic RNAs have functional ORFs. If the translation start

codon was in phase with that of the 166 kDa protein, antibody made to a carboxyl terminal sequence should react with the proteins made from the 3.6 kb and 3.0 kb RNAs.

A structural domain chosen for each peptide included a alpha helix : beta turn motif. This structural motif is common among membrane proteins (123). This motif was chosen because it is generally found at the surface of the membrane protein and is generally highly immunogenic. The sequences corresponding to the structural domain chosen were determined manually and checked on a VAX computer using secondary structure computer programs such as Chou-Fasman (124), Garnier (125), Osguthorpe and Robson (126), Hopp and Woods (127), and Gribskowv, Burgess, and Devereaux (128). In addition, selections were checked on a Parker and Hodges (129) analysis computer program which locates hydrophilic amino acids reside on the protein surface and are probably immunogenic. Once a structural consensus prediction for alpha helix: beta turn was seen using the different predictive computer programs, this sequence was chosen.

A consensus sequence of ten amino acids, **E K P F I E F R E F**, was added to the amino terminus of each peptide in addition to the viral amino acids. This sequence contains a T cell helper epitope. Its presence confers greater antigenicity to the peptide and is only recognized in BALB-C mice (Dr. Marc Koolen, personal communication). The following peptide sequences were synthesized:

Virus Protein	Peptide sequence	Location on the protein
166 kDa	NH_3- E K P F I E F R E F G E L K K C Q D S T - $CONH_2$	nts 4263-4293; carboxyl terminus, ORF 1
24 kDa	NH_3- E K P F I E F R E F K E Y T L D N T T R N S Y - $CONH_2$	nts 4732-4768; middle domain, ORF 2
12 kDa	NH_3- E K P F I E F R E F G D N S P N L G S R-$CONH_2$	nts 5218-5221, 5341-5371 carboxyl terminus, ORF 3
8 kDa	NH_3- E K P F I E F R E F K L D A E T I K A-$CONH_2$	nts 5566-5590; carboxyl terminus, ORF 4

2. Synthesis of Peptides

The four peptides were synthesized on a Milligen 9050 peptide synthesizer. A 0.2 mmole synthesis was done. The PAL resin (130) and glass beads were placed in a column. The glass beads allowed room for the growing peptide minimize pressure problems. Upon completion of synthesis, the column containing the resin-peptide and glass beads was removed.

3. Dichloromethane (DCM) wash of resin-peptide

The resin-peptide and glass beads were washed from the column with DCM and decanted into a glass filtration apparatus with a side arm. The glass beads were rinsed two more times and the liquid was decanted. A water vacuum line was used to pull out the DCM. The opening on the glass apparatus was covered with parafilm, with small holes, and dried under vacuum in a dessicator for at least four hours to remove all traces of DCM for effective cleavage.

4. Purification of Milligen-synthesized peptides after cleavage from the resin

Day 1

Reagent R (0.5 ml thioanisole, 0.3 ml ethanedithiol, 0.2 ml anisole, and 9.0 ml trifluroacetic acid (TFA)) was added to the resin-peptide and stirred gently for two hours at room

temperature to effect cleavage. The glass apparatus was insulated from the heat of the stirrer. The cleavage was completed at 2 hours and the wash steps were initiated. The glass apparatus was tipped over to its funnel side and washed with ten ml portions of TFA four times. All the liquid was caught in a round bottom flask. The peptide, now cleaved from the resin was washed from the resin with TFA. The sample volume was then reduced by rotoevaporation. The liquid was placed in several 30 ml Corex tubes and cold ether was added (around 2/3 full). Five to ten volumes of ether was added to one volume of the TFA-peptide. The tubes were incubated at - 20 degrees overnight.

Day 2

The crude peptide was washed five times to remove organic by-products with 20 ml of cold ether. The Corex tubes were spun for 20 minutes at 4 degrees. The pellets were pooled into one or two Corex tubes, placed into a dessicator and dried under vacuum.

Day 3

The crude peptide was weighed and divided into portions of 200 mg or less.

Crude peptide weight:

Virus Protein	Weight of peptide recovered
166 k	415 mg
24 k	425 mg
12 k	193 mg
8 k	300 mg

The peptides were processed through a Sephadex G-25 column in 0.1 M acetic acid. The fractions were lyophilized overnight.

Day 4

An analytical HPLC run on each peptide was carried out using standard reverse-phase conditions. A Waters Bondapak C 18 column (0.39 x 15 cm) was used to fractionate each peptide. The solvent system used:

Solution	Composition
A	0.1 % TFA in water
B	0.1 % TFA in acetonitrile

The solvent system was used at a flow rate of 1 ml/min with a gradient of 90 % A/10 % B to 45 % A/55 % B over a 45 minute interval. The largest peak was collected and lyophilized. One microgram of the pellet was used for mass spectroscopic analysis.

Day 5

Mass spectroscopy analysis showed the correct mass for each peptide except for the 24 k peptide (figure 5).

Mass spectroscopic results:

Peptide	Calculated Molecular Weight	Mass Spectroscopy Results
166 k	2687.071	2687.0
24 k	2928.131	2910.0
12 k	2338.557	2338.0
8 k	2310.653	2311.0

Day 6

A preparative HPLC purification (131) was done on the remaining peptide. The conditions employed were scaled-up from previously determined analytical HPLC conditions. Fractions were collected and mass spectroscopy was done to determine the fraction containing a homogenous peptide of correct mass. A

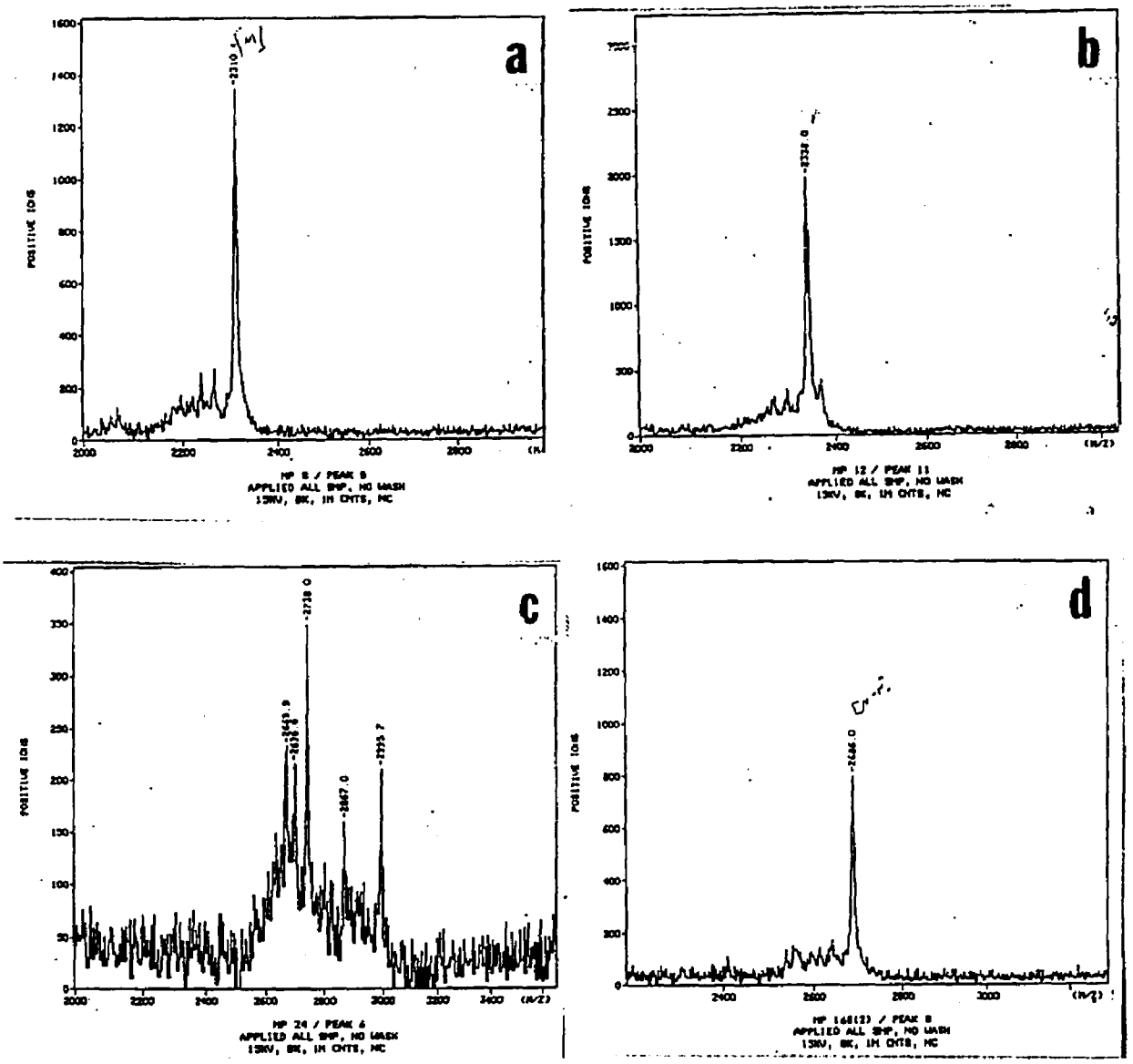


Figure 5. Mass spectroscopic analysis of peptides synthesized for the detection of potato virus X 8 kDa (ORF 4) (a), 12 kDa (ORF 3) (b), 24 kDa (ORF 2) (c), and 166 kDa (ORF 1) (d) proteins.

Waters Bondapak C 18 column (19 x 150 cm) was used with the same gradient conditions as in the analytical HPLC except at a flow rate of 10 ml/min was used.

5. Immunizations and Antigen Preparation

BALB-C mice (two mice/antigen) were immunized by intraperitoneal injections containing one milligram of peptide emulsified in 0.25 milliliters of phosphate buffered saline, one drop of DMSO, and 0.25 milliliters of Freund's complete adjuvant. Antisera was collected 10 days after the priming injection. The titer of each antisera was tested by peptide ELISA (132). Booster injections containing 0.5 milligram of peptide, one drop of DMSO, 0.25 milliliters of phosphate buffered saline (PBS), and 0.25 milliliters of Freund's incomplete adjuvant were repeated two times at two week intervals. The titer of the antisera was checked again by peptide ELISA. It was 1:100,000, so the mice were anesthetized and terminally bled. The blood was allowed to sit at room temperature for two hours and centrifuged at 7,000 rpm for ten minutes. The antisera was recovered in the supernatant.

6. Peptide Elisa

Protocol: Dot Blotting of Peptide Samples

A sheet of nitrocellulose was cut to fit the size of the manifold and pre-wet in 1X PBS prior to its placement in the manifold. The peptide samples (30 ug in 50 ul) were prepared in 1X PBS. The samples were pipetted into the wells under aspiration. The nitrocellulose was removed and air-dried. The membrane was incubated in a 0.8 % glutaraldehyde-PBS solution for 12 minutes while rocking. The membrane was washed four times for 15 minutes in 150 mls Tris/Glycine (2.4 g Trisma base, 30.0 g glycine, 2 liters of water). The membrane was blocked in 5% Blotto (nonfat dry milk in 1X PBS) for at least 30 minutes at 37 degrees centigrade. The membrane was incubated for 90 minutes with immune test sera in (1:10, 1:100, 1:1000, 1:10,000, 1:100,000),

while rocking, in 5% Blotto. The membrane was washed for 10 minutes with 100 mls Tris/saline buffer, washed twice in 150 mls Tris/saline with 0.1 % NP 40, and a final wash in 100 mls Tris/saline buffer. It was then incubated in secondary antibody. In this case it was goat anti mouse IgG-peroxidase conjugate (1:1000 dilution in Tris/saline or blotto). The membrane was washed in Tris/saline two times, twice in Tris/saline with 0.1 % NP40, and once more in Tris/saline buffer. The membrane was blotted with Whatman paper and incubated in the substrate solution (solution A: 15 mgs 4-chloro-1-napthol, 5 mls cold methanol: solution B: 25 ml Tris/saline, 15 mls hydrogen peroxide; pour solution B into A and mix well right before using) until the color reaction is fully developed. The membrane was blotted dry and stored under a transparency to prevent fading and tearing.

7. Dot blot hybridization (Yukio Shirako, personal communication)

Sample preparation:

Fresh leaf tissue (0.5 g) was pulverized in a mortar with a pestle in one milliliter of extraction buffer (10 mM DTT, 1 mM PMSF, 1 mM hexamethyl benzoic acid (HMB), 2 X STE pH 8.0). The sample was frozen at -80 degrees centigrade. Prior to use, the sample was boiled in 1 % sodium dodecyl sulfate for five minutes and spun in microfuge at 15,000 rpm for five minutes.

Protocol:

The nitrocellulose was cut to fit the size of the manifold and pre-wet in TTBS. The wells were pre-wet with 200 ul of TTBS (20 mM Tris-Cl, pH 7.5, 0.15 M NaCl, 0.05 % tween 20). A vacuum was pulled and 0.5% glutaraldehye in TTBS was added to each well and incubated for 15 minutes. Glutaraldehyde (0.5 % in TTBS) was applied to the wells prior to loading sample because proteins less than 15 kDa do not bind efficiently to membranes. The glutaradehyde cross-links the protein to the membrane acting as a

fixative (133). A vacuum was pulled, and the samples were added and incubated for 90 minutes. The top of the manifold was sealed with plastic wrap during the incubation to prevent drying. A vacuum was pulled and the wells were rinsed twice in TTBS (200 ul/well). The membrane was removed from the apparatus while a vacuum was being pulled. It was washed once in TTBS for 5 minutes. The membrane was incubated in a 1:2000 dilution of the primary antibody for 2 hours. The membrane was washed three times in TTBS for 5 minutes each. The membrane was incubated in the secondary antibody (goat anti-mouse IgG conjugated with alkaline phosphatase; 1:2000) for 1 hour. The membrane was washed twice in TTBS and once in alkaline phosphate buffer (AP 9.5) (0.1 M Tris-Cl pH 9.5, 0.1 M NaCl, 5 mM $MgCl_2$) for 5 minutes each. The membrane was incubated in the color reaction. Double-distilled water was used to stop the reaction.

8. Western Analysis

Fresh leaf tissue (0.5 g) was ground in a mortar with a pestle in 5 ml of extraction buffer (10 mM DTT, 1 mM PMSF, 1 mM HMB, 2X STE pH 8.0). The sample was frozen at -80 degrees centigrade prior to use. One ml of the sample was boiled in 2 % SDS for five minutes and spun in a microfuge tube for five minutes. The sample was refrozen at -80 degrees centrigrade and lyophilized. The solid sample was resuspended in 0.5 ml of extraction buffer and ten microliters was loaded into the wells of a mini-Laemmli polyacrylamide gel (5 % stacking, 12.5 % resolving). The proteins were separated at 70 volts for 4 hours at room temperature. The gel was carefully removed and soaked in transfer buffer for ten minutes. Gel strips were cut and nitrocellulose (0.45 um) strips were placed over the gel strips and placed on a transfer screen

which had been pre-soaked in transfer buffer (14.5 g Tris base, 67 g glycine pH 8.0 in 4 liters of water; add 1200 ml methanol and bring final volume to 6 liters with water). The nitrocellulose strips were allowed to transfer for 2.5 hours (8 kDa protein), 3 hours (10 to 90 kDa proteins) and 6 hours (150 to 180 kDa proteins) at 40 volts and 4 degrees centigrade. The western analysis protocol was performed as described in the protocol for dot blot western using alkaline phosphatase.

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CHAPTER III. RESULTS

Electron Microscopic Examination of PVX and other Potexviruses

A lyophilized virus sample of PVX (ATCC strain) was resuspended in 50 mM phosphate buffer, pH 7.0 and inoculated onto Carborundum-dusted leaves of two month-old *Nicotiana glutinosa* plants. The virus was harvested two weeks post inoculation. A virus isolation procedure specifically designed for PVX to prevent virus aggregation and fragmentation was followed (55). Drops of partially purified virus preparations were examined by quick dip with the electron microscope. Seven different particle sizes were detected along with a minor population of random size particles (figure 6). A histogram of the size distribution of PVX particles is shown in figure 7. The black boxes are measurements of large populations of discrete, reproducible size classes of virus particles. The white boxes are measurements of a heterogenous size distribution of minor particle species comprising 5-10 % of the total particle population which may represent fragmentation products of the virus purification method. A replica grating grid was used to measure the particle lengths. The particle lengths of PVX were measured as follows: full-length particles - 515 nm, smaller particles - 290 nm, 275 nm, 170 nm, 140 nm, 110 nm and 75 nm. The number and sizes of the particle lengths correspond to the number and sizes of viral RNAs previously reported (68).

Figure 6. Electron microscopic examination of a quick dip preparation of partially purified ATCC potato virus X particles.


ATCC
80 nm

Figure 7. Electron microscopic examination of partially purified particles of ATCC potato virus X by serologically specific electron microscopy (SSEM) (a) and the particle size distribution of ATCC potato virus X (b).

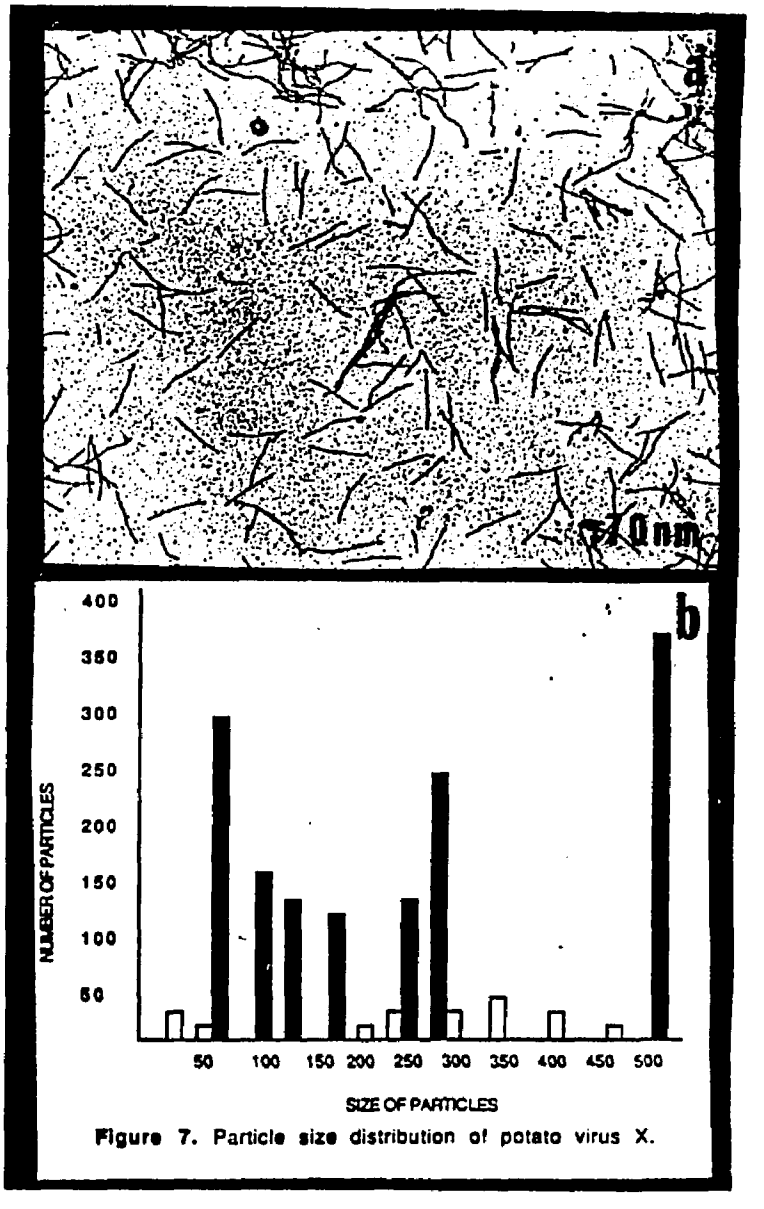


Figure 7. Particle size distribution of potato virus X.

The different sizes of ATCC PVX were surprising since it is defined as a flexible rod of uniform length (2). Therefore, it was important to determine if this particle morphology was unique to PVX or common to other potexvirus members. The potexvirus members chosen for electron microscopic examination include: PMV, NMV, WCIMV, and CCMV. PMV, NMV, and WCIMV were chosen because they are often compared to PVX in the literature and were easily obtainable. CCMV was chosen because it is a tentative member of this group and has yet to be characterized.

Lyophilized samples of PMV, NMV, WCIMV, and CCMV were resuspended in 50 mM phosphate buffer pH 7.0 and inoculated onto Carborundum-dusted leaves of two month old papaya, *Nicotiana benthamianum, Pisum sativum*, and cassava respectively. The viruses were isolated two weeks later using the same isolation method as the one used for PVX (55). A quick dip preparation of the isolated virus preparations was prepared for electron microscopic examination. Different particle sizes were also observed indicating that the potexvirus particle morphology as described in the literature (ie. a single, full-length particle (3, 4, 5, 6)) may be inaccurate.

Different sizes of ATCC PVX, PMV, NMV, and WCIMV were shown in electron micrographs from earlier literature (64, 65, 66, 67), but the smaller sizes were ignored. In the present investigation, the

smaller particles were examined to determine if they were contaminating viruses, a host effect, purification artifacts, a strain effect, defective interfering particles, satellites, and/or encapsidated subgenomic RNAs.

To address the possibility of contaminating viruses, antisera prepared to the coat proteins of PVX, PMV and WCIMV were obtained for use in serologically specific electron microscopy (SSEM) (110) and decoration experiments (111). NMV and CCMV were not examined because antisera for each was unavailable. Two negative controls were used in these experiments: (1) healthy leaf extracts were incubated on PVX, PMV, or WCIMV antisera (panels A, B, and C in figure 8, and (2) PVX, PMV, and WCIMV virus preparations were incubated with an unrelated plant virus antisera (ie. antisera made to the coat protein of TMV) (panels D, E, and F).

The results of SSEM experiments for ATCC PVX, PMV, and WCIMV indicate specific attachment of seven, six, and seven discrete particle sizes respectively (figures 7, 9). Random size distributions of smaller particles were also detected. Since antisera for NMV and CCMV were unavailable, each was examined by a quick dip preparation (figure 9). Histograms of particle size distributions of PMV, NMV, WCIMV, and CCMV are shown in figure 10. The black boxes are measurements of the predominant virus particle species and the white boxes are measurements of minor particle populations.

Figure 8. Negative controls of serologically specific electron microscope experiments. Panels A, B, and C - healthy leaf extracts were incubated on PVX, PMV, and WClMV antisera, respectively. Panels D, E, and F- partially purified PVX, PMV, and WClMV particles were incubated on TMV antisera.

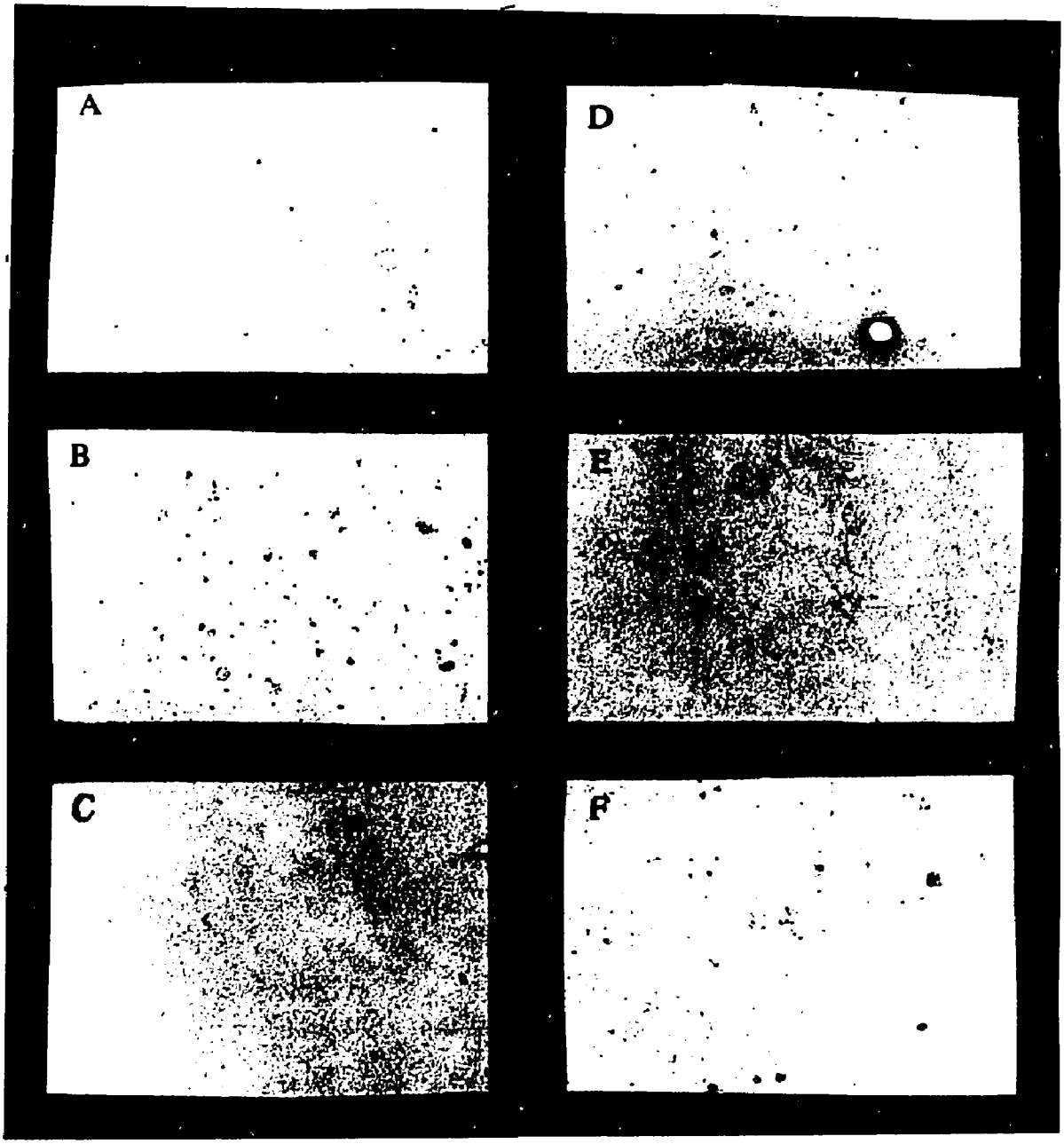

A
D
B
E
C
F

Figure 9. Electron microscopic examination of papaya mosaic virus (PMV) and white clover mosaic virus (WClMV) by serologically specific electron microscopy, and narcissus mosaic virus (NMV) and cassava common mosaic virus (CCMV) by quick dip preparations.

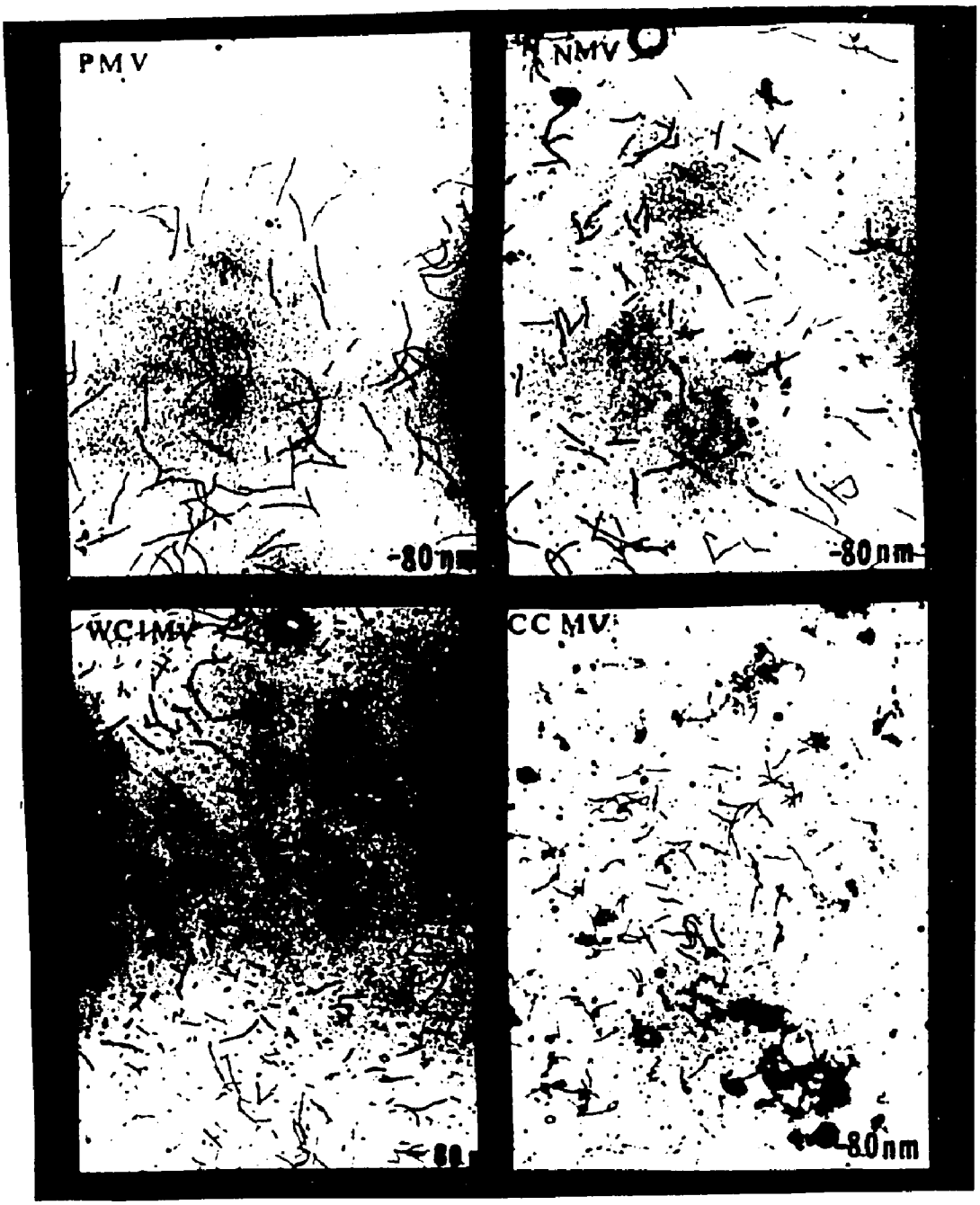
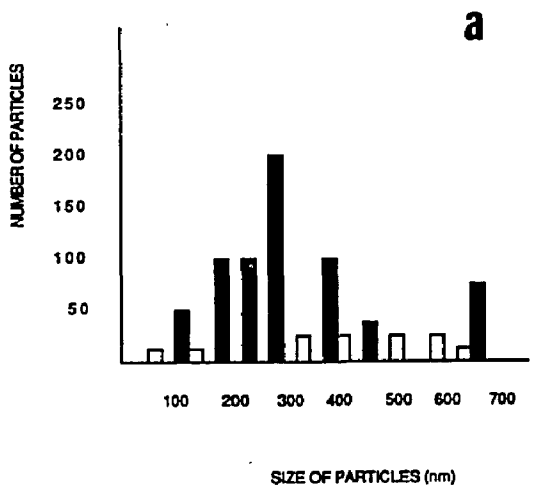
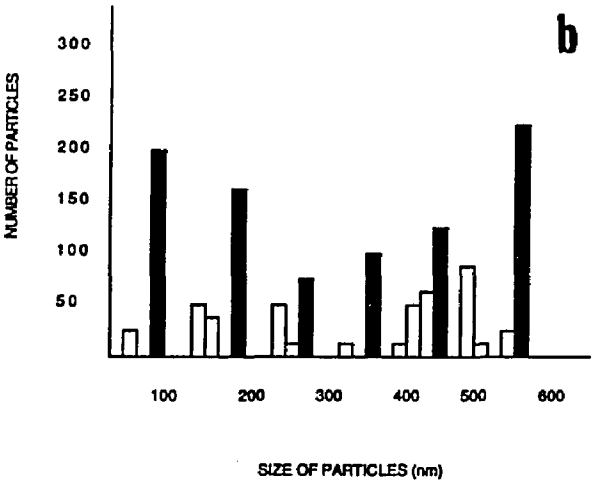
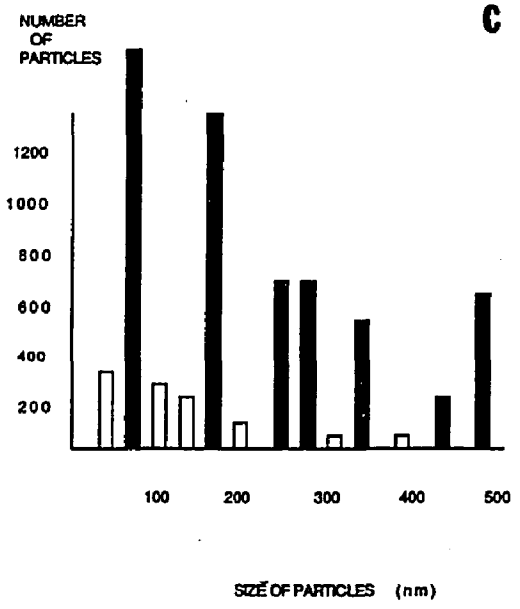
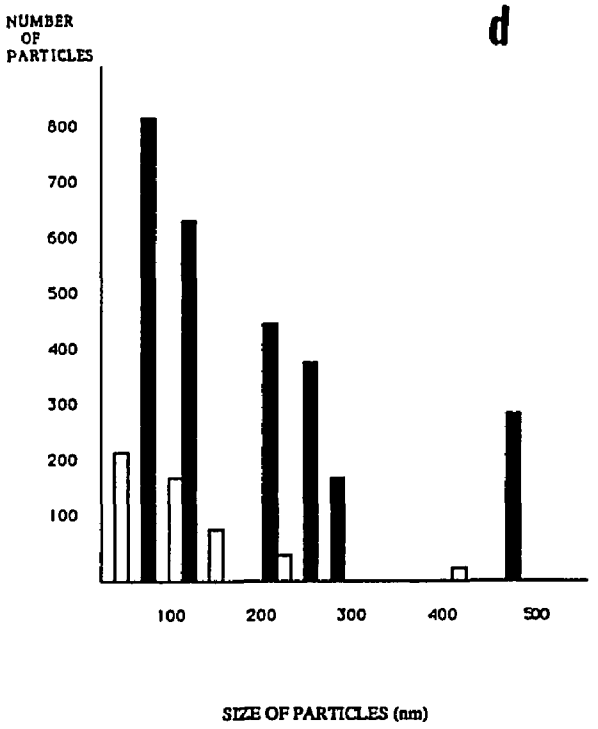

PMV
80nm
NMV
80nm
WCIMV
CC MV
80nm

Figure 10. Particle size distributions of papaya mosaic virus (a), narcissus mosaic virus (b), white clover mosaic virus (c), and cassava common mosaic virus (d).


a
NUMBER OF PARTICLES
250
200
150
100
50
100
200
300
400
500
600
700
SIZE OF PARTICLES (nm)


b
NUMBER OF PARTICLES
300
250
200
150
100
50
100
200
300
400
500
600
SIZE OF PARTICLES (nm)


c
NUMBER OF PARTICLES
1200
1000
800
600
400
200
100
200
300
400
500
SIZE OF PARTICLES (nm)

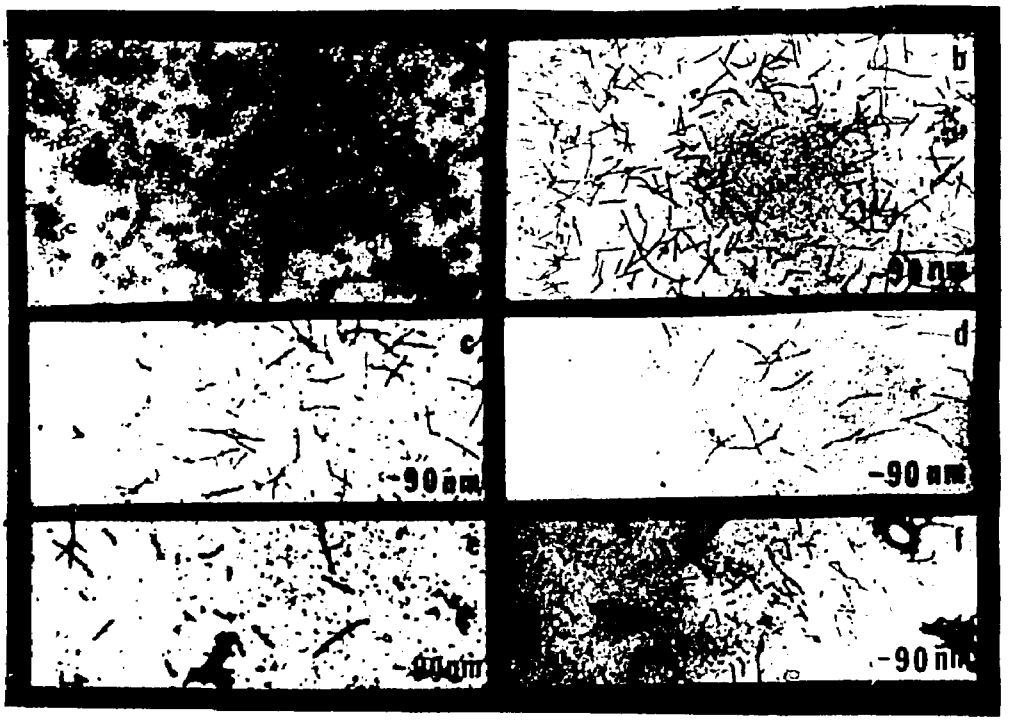

d
NUMBER OF PARTICLES
800
700
600
500
400
300
200
100
100
200
300
400
500
SIZE OF PARTICLES (nm)

Particle lengths of PMV were measured: full-length particles - 530 nm, smaller particles - 380 nm, 300 nm, 170 nm, 110 nm and 70 nm. The predominant particle lengths of WCIMV were measured: full-length particles - 480 nm, smaller particles - 430 nm, 340 nm, 270 nm, 240 nm, 180 nm, and 80 nm. Calculated particle sizes of NMV include: full-length particles - 550 nm, smaller particles - 480 nm, 380 nm, 270 nm, 210 nm, 180 nm, and 80 nm. Particle lengths of CCMV were measured: full-length - 495 nm, smaller particles - 297 nm, 248 nm, 220 nm, 124 nm, and 74 nm.

Decoration experiments for ATCC PVX, PMV, and WCIMV indicate that the smaller particles have a beaded appearance showing serological reactivity to its own antisera (figure 11). In the right panels of each figure, PVX, PMV, and WCIMV particles did not have a beaded appearance when each was reacted to TMV antisera. In the left panel of each figure, PVX, PMV, and WCIMV particles had a beaded appearance when each was reacted with its own antisera. The smaller particles of PVX, PMV, and WCIMV shared no serological identity with TMV antisera, but did react with their own suggesting that the smaller particles were not contaminating viruses.

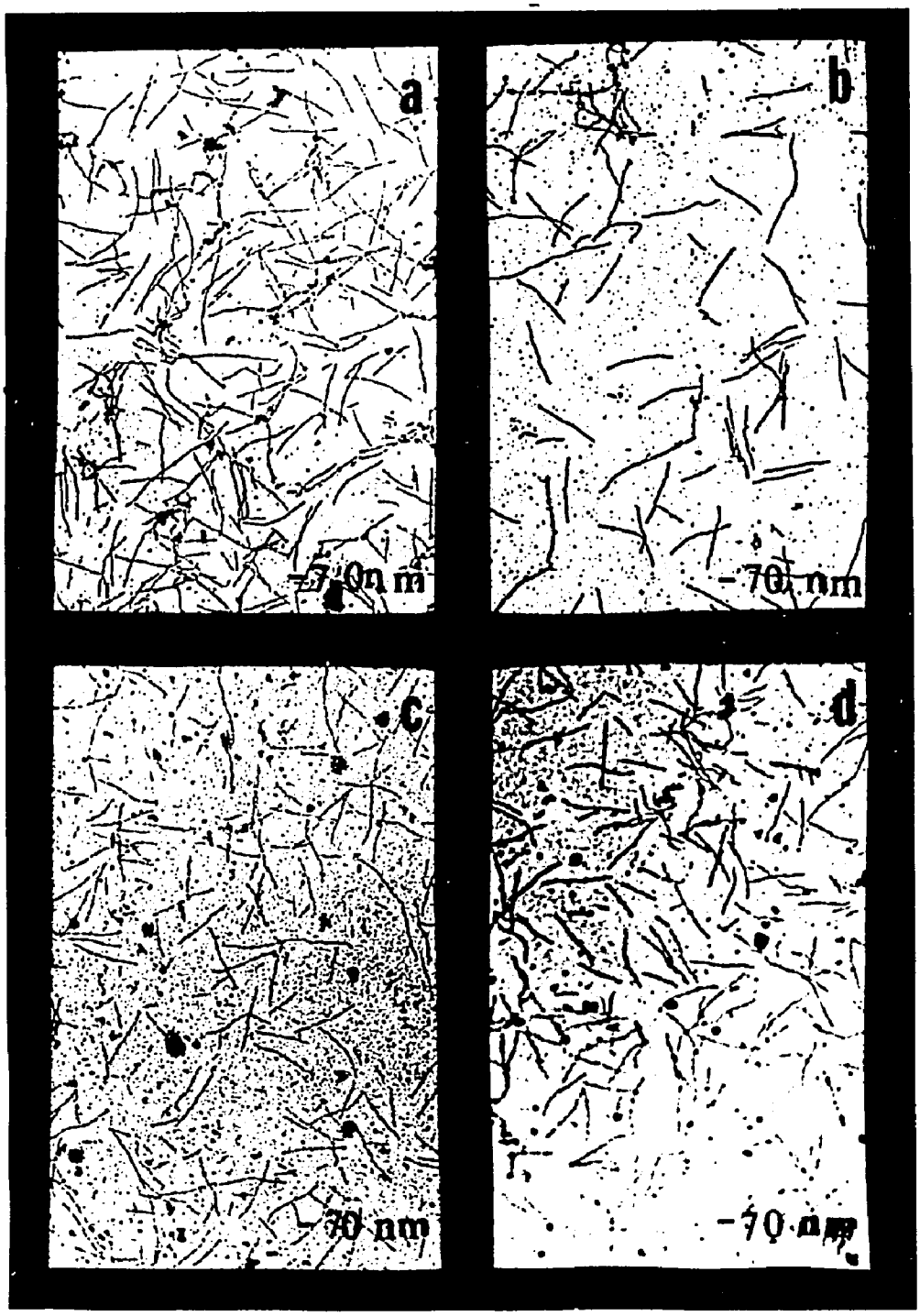
Two additional factors which might affect the number of virus particle sizes include the host species for propagation of the virus and purification procedure used for virus isolation. To examine these possibilities, different tobacco varieties, *Nicotiana glutinosa, N.*

Figure 11. Examination of partially purified virus particles, potato virus X (a), papaya mosic virus (c), and white clover mosaic virus (e) with negative controls (b, d, f) by the electron microscope technique of decoration.


b
-90 nm
c
-90 nm
d
-90 nm
e
f
-90 nm

tabacum var. Xanthi, N. tabacum var. Samsun and other hosts *Datura stramonium and Lycopersicon esculetem* were inoculated with ATCC PVX. PMV and NMV were inoculated onto *Datura stramonium* and *N. benthamianum.* WCIMV was propagated in *Datura stramonium* and *Pisum sativum.* Six different viral purification methods using different buffers (phosphate, Tris, citrate, borate, or HEPES) at different pH (7.2, 9.0, 8.2, or 7.5) and molarities (0.2, 0.1, 0.5, or 0.01) were used to isolate PVX. In two methods organic solvents were used, butanol (114) in one and butanol/chloroform (52) in the other. Virus was recovered by different manipulations post homogenization: differential centrifugation (52), PEG (53), charcoal (50), acidification/$AgNO_3$ /PEG (55) or Triton X/PEG (114). In still another method, infected leaf tissue was homogenized by grinding in a mortar with a pestle and virus was separated from cellular components by DEAE chromatography (51). The number of different virus particle sizes isolated were similar regardless of the purification method used (figure 12) In figure 12, PVX virus was purified from *Nicotiana glutinosa* and *Nicotiana tabacum* var. *Samsun* two weeks post infection by the acidification/AgNO /PEG method (55). In each host, multiple virus particles were observed. In figure 12, the virus particle morphology was compared using the charcoal method (50) and the butanol/Triton X/PEG method (114). In each case, PVX was propagated in *Nicotiana glutinosa* plants and isolated two weeks post inoculation. A quick dip preparation of the purified virus preparations isolated from different hosts and by

Figure 12. Electron microscopic comparison of potato virus X isolated from infected *Nicotiana glutinosa* and *Nicotiana tabacum* var. *Samsun* (a) and the comparison of its purification by charcoal and butanol-triton-X-PEG from infected *Nicotiana glutinosa* (b).


a
b
c
d
70 nm
70 nm
70 nm
70 nm

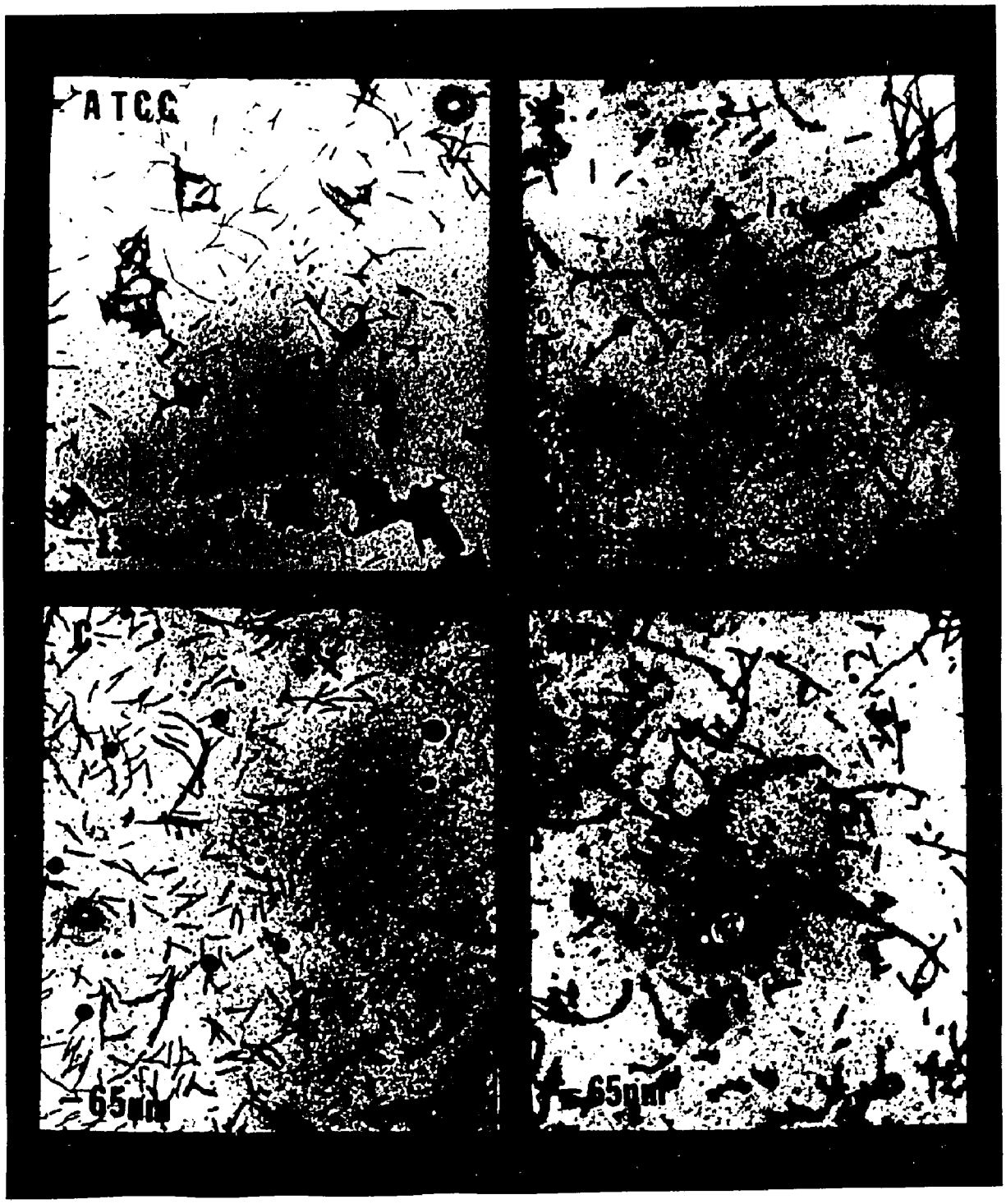
different purification methods was examined.

Two different methods were used to purify PMV, NMV, and WCIMV from the hosts listed above. One method involved charcoal clarification (50) and the other used butanol-Triton X-PEG (114). The virus particle morphology from both methods was similar.

It is known that some strains of TMV do not encapsidate their subgenomic RNAs (77, 78). To determine if the different particle sizes were unique to this PVX ATCC strain, crude extracts and purified preparations of three other PVX strains (C, G, HB) were examined with SSEM and identical results were obtained suggesting that this was not a strain effect (figure 13).

The three remaining possibilities to be addressed included: defective interfering (DI) particles, satellites, and encapsidated subgenomic RNAs. DI particles contain truncated portions of genomic RNA. These smaller RNAs interfere with the replication of the genomic RNA from which it is derived (134). Satellites, on the other hand, are small virus particles found in association with a particular virus (helper virus) (135). The satellite may or may not share sequence homology with its helper genomic RNA. It may encode its own coat protein. If it does not, it is encapsidated by its helper virus (135). Even though the specificity of SSEM and decoration indicates that contaminating viruses are not present,

Figure 13. Electron microscopic examination of PVX strains, ATCC, C, G, and HB.

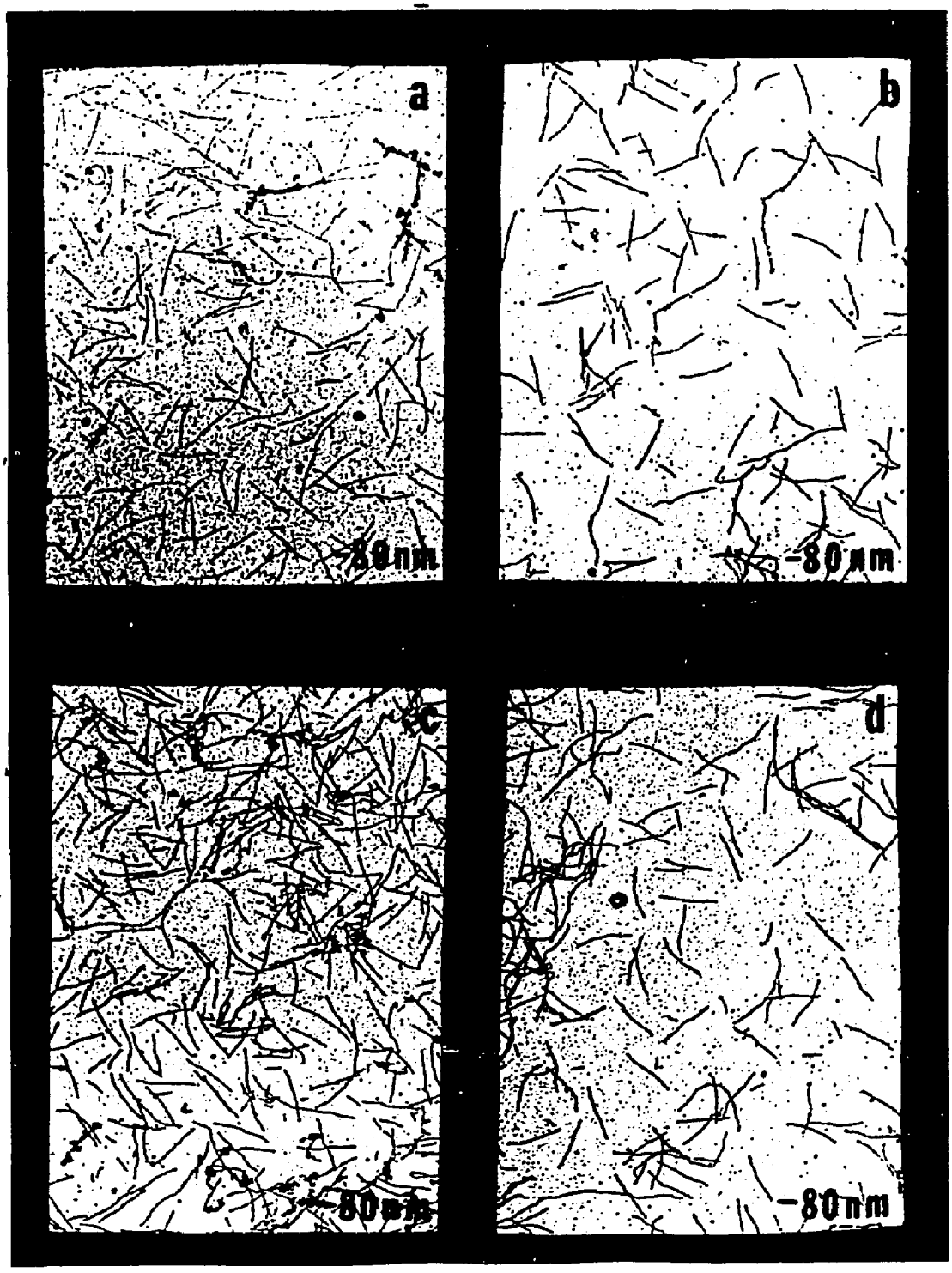

ATCG
65μm
65μm

each PVX isolate was inoculated onto its local lesion host, *Gomphrena globosa*. This provides clonal isolation of the PVX isolate. In addition, a single local lesion was ground up for SSEM. Multiple particle sizes were still observed. If the smaller particles of PVX were DI particles or satellites, they would have been lost during passage through the local lesion host of PVX. The continued presence of smaller particles within the local lesion and propagation in systemic hosts indirectly supports the conclusion that the smaller particles are encapsidated subgenomic RNAs.

The simple process of cellular maceration has been previously reported to minimize fragmentation that may occur during virus purification (78). To determine if the smaller particle sizes observed in purified virus preparations were breakage products, the particle morphology from crude extracts was compared. Infected leaf tissue from PVX, PMV, NMV, and WCIMV was ground in a mortar with a pestle in buffer and SSEM was done. In each case, the particle morphology remained unchanged indicating that the smaller particles were not host dependent or purification artifacts (figure 14).

Since previous literature indicated that only full-length genomic PVX virus particles of uniform size have been seen with the electron microscope, it was an important control to obtain such an isolate from a lab which has published this observation. Two such strains,

Figure 14. Electron microscopic comparison of ATCC potato virus X isolated from crude extracts (a) and a partially purifed virus preparation (b) and examination of strains Robert's (c) and V349 (d) by serologically specific electron microscopy.


a
b
c
d
80nm
80nm
80nm
80nm

V-349 (Shepherd isolate) and Robert's PVX (Purcifull isolate) were obtained, clonally purified in the local lesion host, *G. globosa,* and propagated in *N. glutinosa.* A single local lesion from each isolate was ground in buffer and inoculated onto *N. glutinosa.* Virus was harvested two weeks later and SSEM was done. All seven virus particle sizes were observed (figure 14).

II. Examination of Virus Purification and RNA Extraction Procedures

In the study of the replication strategy of a virus, it is important to isolate intact particles free of host material and to isolate intact viral nucleic acids. The method used to purify virus is important in the attainment of this goal. If proteases or ribonucleases are present in moderate levels, they can affect all subsequent steps necessary to delineate an appropriate model. Another problem that is often overlooked is the methodology chosen can be unintentionally biased favoring a particular replication strategy. This was the case with TMV (56).

In the present investigation, PVX virus was purified from protocols developed in the early 1900's to the most recent method in 1988 (36, 51, 55), and multiple particle sizes were always detected. However, upon RNA extraction of purified particles, a single RNA species was detected. The absence of smaller RNAs extracted from

purified virus preparations was possibly due to one or more reasons:

1. The RNA extraction methods (136, 137, 138) commonly used may be inadequate to allow detection of the smaller RNAs. Typical extraction methods employ 1 % SDS, low salt conditions, high virus concentrations (1-10 mg/ml), and vortexing in phenol or phenol/chloroform for 30 seconds. Since purified PVX preparations are insoluble, dissociation under typical conditons may be incomplete after phenol extraction resulting in partial extraction of viral RNA.

2. The age of the leaf tissue harvested did not contain large quantities of the smaller RNA species (subgenomic RNAs).

Both reasons probably contributed to the absence of detectable subgenomic RNAs from purified virus preparations over the years PVX has been studied.

PVX research has progressed slowly because a virus purification method has not been developed that gives high yields of purified PVX particles with constant properties (49). When PVX was concentrated by salt or acid precipitation, low or high speed centrifugation, purified virus preparations formed insoluble rope-like complexes. These complexes were still infectious and serologically active, but posed serious problems for further structural studies and RNA

analysis. Most reliable data on the shape, size, and size distribution of PVX particles came from electron microscopic examination of untreated sap (47). The insolubility is due to the aggregation of virus particles into thread-like structures with host cell constituents adhered to its surface.

In the present investigation, the purified virus preparations derived from other protocols did not result in satisfactory isolation of the smaller viral RNA species. The genomic RNA was the only species reported in previous literature (2). Based on the rod length-frequency data obtained by electron microscope observation of virions, the absence of subgenomic RNAs was puzzling. Different viral RNA purification procedures specific for the isolation of PVX RNA (37) and other viral RNAs (136, 137, 138) from purified virions was tried. The results were always the same: a single, full-length genomic RNA was seen. To explain this discrepancy, experiments were designed to address the possibility that due to extensive secondary structure, the subgenomic RNAs were in an aggregated state that resulted in their co-migration with full-length RNA. Denatured agarose gels containing formaldehyde or glyoxyl (117, 139) were prepared and the virion RNA preparations migrated as a single high molecular weight band contradicting this assertion.

Purified virion RNA preparations were routinely separated by electrophoresis through agarose gels. To test the possibility that

perhaps the agarose matrix was too porous for adequate resolution of the viral RNA mixture, purified virion RNA preparations were subjected to electrophoresis in a 3.5 % polyacrylamide-0.5 % agarose composite gel. Once again only the genomic RNA was detected. In addition, when leaf tissue from PVX-infected plants (showing symptoms) was processed for total RNA isolation, only ribosomal RNAs and/or full-length PVX RNA were detected by ethidium bromide. Dolja, Grama, Morozov, and Atabekov (68) examined PVX RNAs from partially purified virus preparations and total RNA from PVX-infected leaf tissue. The viral single-stranded RNAs isolated from total RNA preparations were visualized by Northern blot hybridizations using a probe containing coat protein sequences while only the genomic RNA was detected from purified virus preparations.

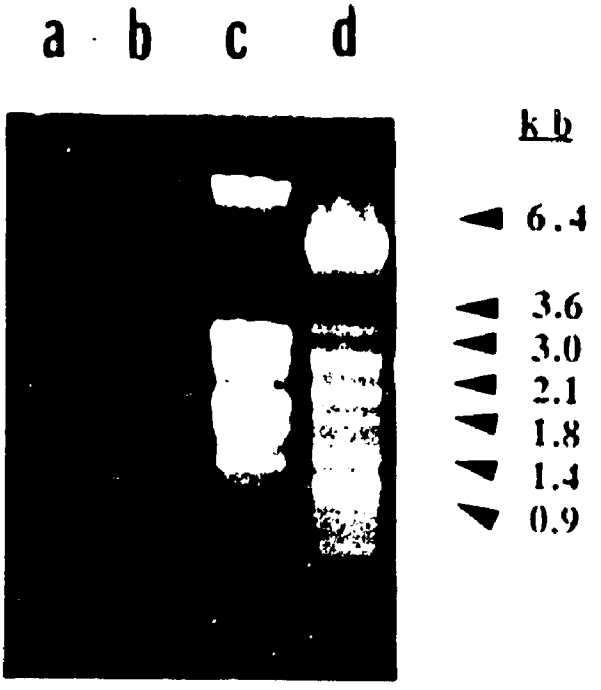
Purified viral RNAs were then treated directly with formaldehyde (140) and loaded onto a 3.5 % polyacrylamide-0.5 % agarose gel. The combination of direct denaturation and its resolution in a polyacrylamide-agarose gel should have effectively separated the subgenomic RNAs from the genomic RNA. The genomic RNA was still the only RNA detected.

Later a virus purification method developed for a plant virus (TEV) in another virus group was tried (114). Smaller RNA species were detected from the purified virions, however, the smaller RNAs

were smeared. The purification method had to be modified to allow reproducible isolation of intact virus particles and viral RNAs. A method was developed (see Materials and Methods) to minimize manipulation of the virus and its exposure to degradative components in the crude extract. The final purified virus preparation still had aggregation, but this problem was addressed by developing an RNA extraction method that could overcome this problem. Higher salt and SDS concentrations, dilution of the purified virus, and longer vortexing in the phenol/chloroform step resulted in the effective dissociation of purified virus clumps which may trap ribonucleases and proteases allowing detection of the subgenomic RNAs. The methodologies developed are adequate for the isolation of PVX viral RNAs and should be suitable for all potexvirus members (figure 15).

The virus purification and RNA extraction procedures were tested on another plant virus from a different virus group with a similar rod-like particle morphology. The virus chosen was tobacco etch virus. It is a flexuous, rod-like virus 725 nm in length encapsidating a single positive-sense 9.5 kb RNA. It is a member of the potyviruses (141). If the virus purification method developed causes extensive fragmentation, then multiple RNAs not necessarily the same size as PVX smaller RNAs should be detected. Upon RNA extraction from TEV virus preparations, a single RNA was isolated. The purified virus preparations were examined on the electron

Figure 15. Analysis of PVX viral RNAs isolated from a partially purified virus preparation. Lane a: healthy control, Lane b: BMV viral RNAs, Lane c: Total RNA extracts (LiCl pellet) isolate from healthy leaves, Lane d: PVX viral RNAs.

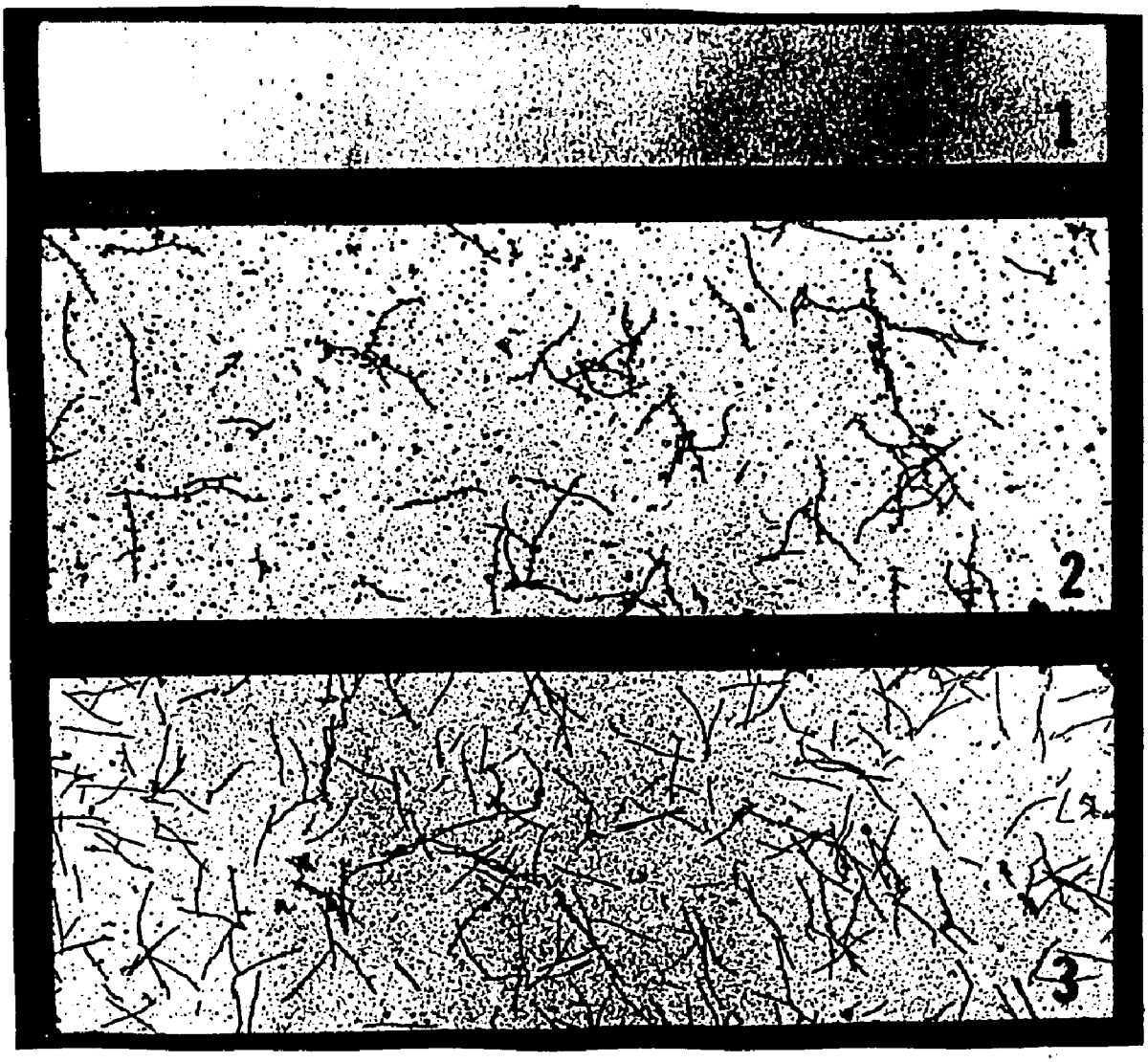

a b c d
kb
6.4
3.6
3.0
2.1
1.8
1.4
0.9

microscope (figure 16). In the upper panel, healthy *Nicotiana* leaves were processed by the same method as a control. In the middle panel, TEV virus particles were purified from *Nicotiana glutinosa* plants two weeks post inoculation, and the lower panel is PVX virus particles extracted from the same host and and at the same time as TEV. Extensive fragmentation of both viruses was not observed (figure 16).

Time course analysis of PVX particle lengths and the RNAs extracted from these particles

Once a virus purification and RNA extraction method produced consistent high quaility RNAs (figure 15), one inconsistency remained. While the sizes of the RNAs were invariant, the number and quantity of the smaller RNAs differed from preparation to preparation. Two variables that might account for this are (1) different hosts of different ages were used for PVX propagation and (2) virus was purified at different time intervals (10 to 14 days) post inoculation. If the smaller RNAs arose from random fragmentation, it was unlikely that reproducible RNA sizes would result from different batches of purified virions. If the fragmentation occurred at "hot" spots along the genomic RNA, consistent numbers and quantities of RNAs should appear. Alternatively, if the smaller RNAs are subgenomic RNAs, the sizes would remain constant, while the quantity and number may vary

Figure 16. Analysis of the virus purification method developed for potato virus X by the isolation of tobacco etch virus. Panel 1: healthy control, Panel 2: partially purified tobacco etch virus particles, and Panel 3: partially purified potato virus X particles.


1
2
3

during infection. One of the advantages of producing subgenomic RNAs is that a plant virus is able to economically express proteins as needed during infection.

The variable number and quantity of smaller RNAs extracted from purified virions were examined by purifying the virus at different times post inoculation. The intervals chosen were 5, 7, 9, 11, and 13 days post inoculation. The virus at each time interval was isolated by the method developed in this investigation and used for SSEM analysis. There was a shift in particle size with the smaller particles predominating at 5 days post inoculation to full-length particles at 13 days post inoculation (figure 17). RNA extraction of the purified virus preparations isolated at 5, 7, 9, 11, and 13 days post inoculation corroborated a shift of six smaller RNA species predominating at 5 days and genomic RNA at 13 days. (figure 18). These results suggested that the smaller virus particles are not fragments of the virus purfication method. Otherwise, similar virus particle sizes and RNA species would have been present at all the time intervals studied. The results also suggest that during infection there is temporal expression of the smaller RNAs. If this is true, it would explain why no one has detected smaller RNAs since others used purified PVX virions 14 days post inoculation or later for their studies (50, 52, 53, 55).

Figure 17. Examination of partially purified particles of potato virus X isolated at 5, 7, 9, 11, and 13 days post inoculation. The numbers in the panel coincide with the days the virus was isolated. Lane H: healthy control, Lanes 5, 7, 9, 11 and 13: partially purified potato virus X particles isolated at 5, 7, 9, 11, and 13 days post inoculation.

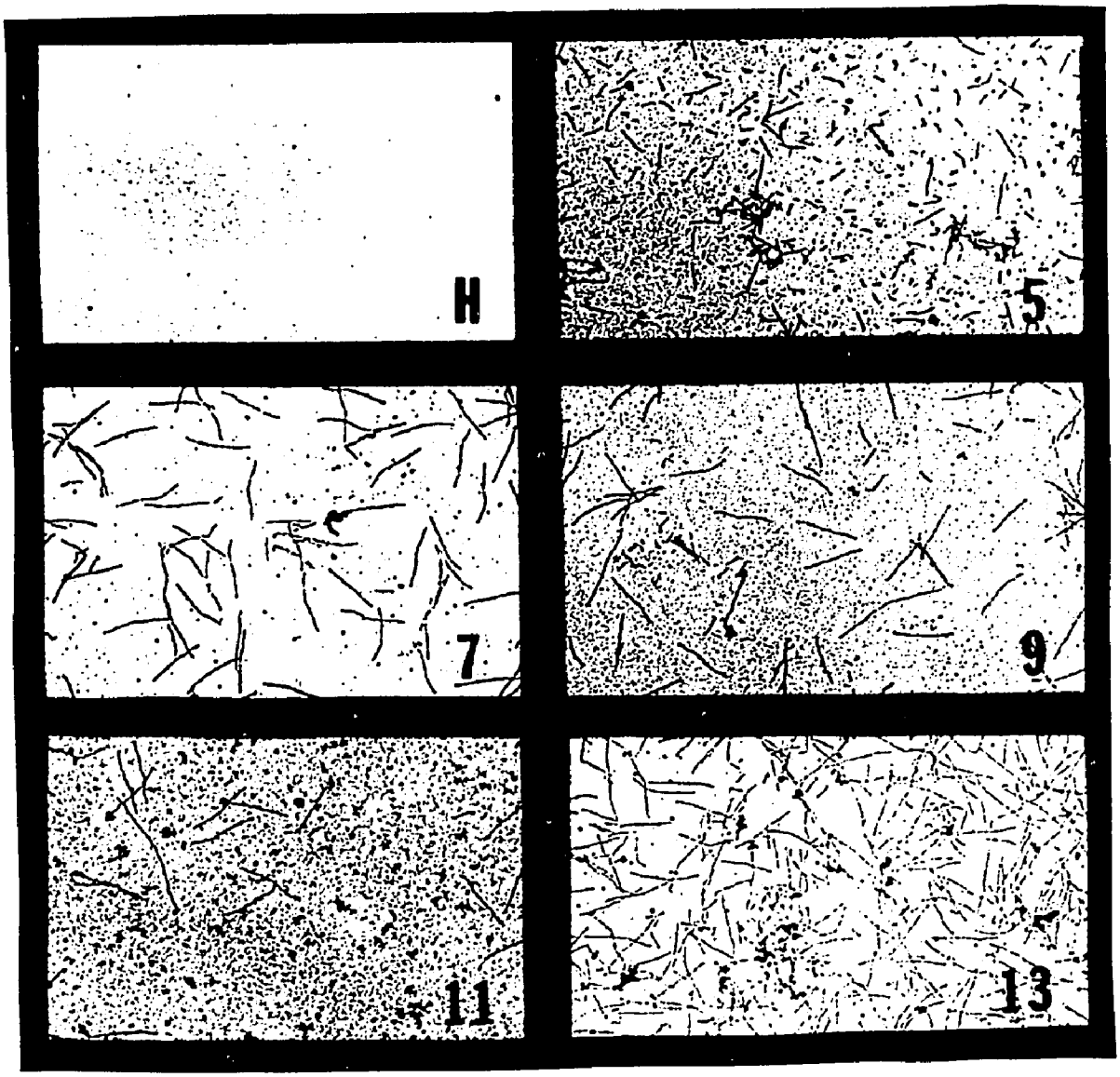
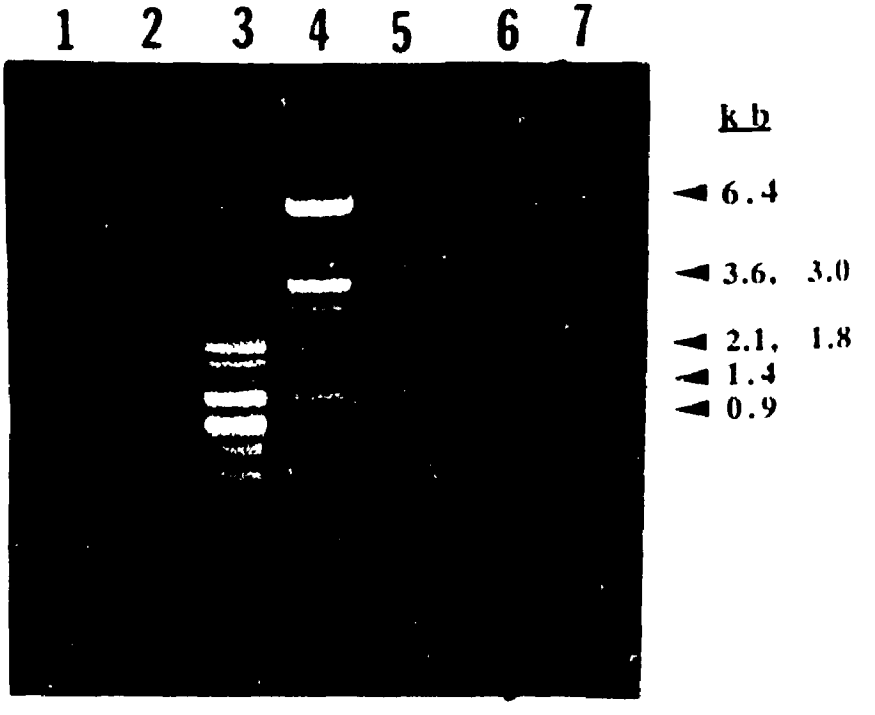

H
5
7
9
11
13

Figure 18. Examination of PVX viral RNAs extracted from partially purified potato virus X particles isolated 5, 7, 9, 11, and 13 days post inoculation by serologically specific electron microscopy. Lane 1: BMV viral RNAs, Lane 2: healthy control, Lanes 3, 4, 5, 6, and 7: PVX viral RNAs extracted from PVX particles isolated at 5, 7, 9, 11, and 13 days post inoculation.


1 2 3 4 5 6 7
kb
6.4
3.6, 3.0
2.1, 1.8
1.4
0.9

Definitive studies on the size distribution of PVX over time were not done since aggregation was still present. There appeared to be consistency between particle size populations and the number and sizes of RNAs extracted. Other virus purification methods in which aggregation was not as extensive, charcoal clarification (50) and acidification/$AgNO_3$/PEG (55), were not used because the smaller RNA species were not detected in the purified virus preparations using these methods.

Characterization of PVX viral RNAs from purified virus preparations

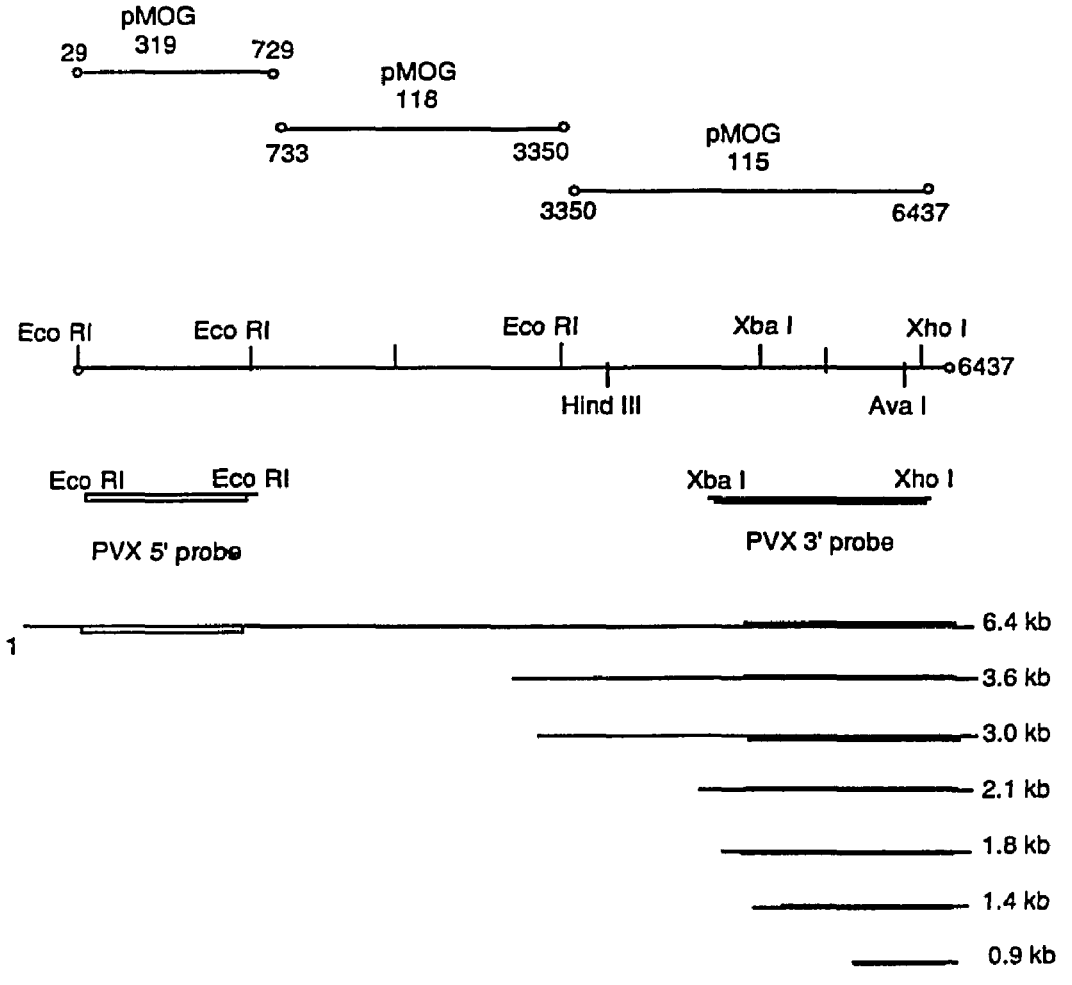
A. Northern blot hybridization analysis to determine if the smaller viral RNA species were 3' coterminal with PVX genomic RNA

Seven RNA species were observed when RNA extracted from purified PVX particles was electrophoresed in denaturing formaldehyde gels (117) or nondenaturing agarose gels. Using genomic TMV and BMV RNAs as standards, the molecular sizes of the seven viral RNA species were estimated to be 6.4 kb, 3.6 kb, 3.0 kb, 2.1 kb, 1.8 kb, 1.4 kb, and 0.9 kb.

One criterion used to determine the authenticity of subgenomic RNAs is the presence of a 3' coterminal sequence and the absence of

a 5' terminal sequence with its genomic RNA. Three PVX clones, pMOG 115, 118, and 319, spanning the entire genome minus the first 28 nucleotides were obtained. pMOG 319 contains 704 bases at the 5' terminus of genomic PVX, but lacks the first 28 nucleotides. pMOG 118 contains PVX genomic bases 733 to 3350 and pMOG 115 contains bases 3350 to 6437. The clones are derivatives of pUC 9 with each PVX sequence inserted into an EcoRI site in the polylinker region. The PVX genomic sequence has been determined (36) and was entered into GENEBANK. A restriction map was obtained (figure 19).

To determine if the smaller RNAs extracted from purified PVX particles were virus-specific, a 704-bp restriction fragment of PVX cDNA from pMOG 319 (EcoRI), beginning at nucleotide 29 and extending to nucleotide 733, was radiolabeled by random prime labeling with ^{32}P-dATP and used as a 5' terminal PVX probe in Northern blot hybridization analysis. Total RNA extracts from healthy and PVX-infected leaf tissue (lanes d, e, f, h), pMOG 118 (EcoRI) and pMOG 115 (EcoRI) restriction fragments (lanes j, k) spanning the middle and 3' termini of genomic PVX, and healthy leaf tissue processed in a similar fashion as infected leaf tissue for virus isolation (lane b) were added as negative controls. pMOG 319 (EcoRI) restriction fragment (lane i) used as a probe in this experiment was added as a positive control. TMV and brome mosaic virus (BMV) RNAs (lane a) were used as molecular weight



Restriction map of PVX cDNA

Figure 19. Restriction map of PVX cDNA. The 5' probe contained nucleotides 29 to 729 of the 5' end of PVX genomic RNA. The 3' probe contained nucleotides 3452-6302 at the 3' end of PVX genomic RNA.

standards. One microgram of viral RNAs and DNA restriction fragments and seven micrograms of total RNA extracts isolated from healthy and PVX-infected leaf tissue were separated by electrophoresis in a 1.0 % denaturing agarose-formaldehyde gel, transferred to a hybridization membrane by capilllary action, and hybridized with a 5' terminal PVX probe (pMOG 319 EcoRI). The upper panel in figure 20 shows an ethidium bromide-stained agarose-formaldehyde gel of viral RNAs, total RNA extracts, and PVX restriction fragments spanning the genome. The lower panel in figure 20 shows an autoradiogram of the same gel in the upper panel hybridized with the 5' terminal PVX probe. One major RNA species, genomic RNA (lane c), was detected. Also detected was the positive control, pMOG 319 EcoRI restriction fragment (lane i). The six smaller RNA species extracted from PVX virions were not detected suggesting that they are not genomic RNA degradation products. No viral RNAs were detected from total RNA extracts isolated from PVX-infected leaf tissue. The failure to detect the genomic RNA may be due to degradation of the viral RNAs during extraction or the titer of the viral RNAs at the time of isolation was too low to be detected.

To determine if the six smaller RNA species were 3' coterminal with genomic PVX, a 2850-bp restriction fragment of PVX cDNA from pMOG 115 (Xba I, Xho I), beginning at nucleotide 3452 and extending to nucleotide 6302, was radiolabeled with ^{32}P-dATP and

Figure 20. Northern blot analysis of PVX viral RNAs separated under denaturing conditions using a 5' terminal cDNA PVX probe. Top panel: A 1.0 % agarose-formaldehyde gel containing: Lane a: TMV, BMV viral RNAs, Lane b: healthy control, Lane c: PVX viral RNAs, Lane d: healthy total RNA extracts (LiCl pellet), Lane e: total RNA extracts from PVX-infected leaf tissue, Lane f: total RNA extracts (LiCl supernatant), Lane h: total RNA extracts (LiCl supernatant) from PVX-infected tissue, Lane i: pMOG 319 (Eco RI), Lane j: pMOG 118 (EcoRI), and Lane k: pMOG 115 (EcoRI). Bottom panel: Northern blot of the gel in the top panel hybridized to the 5' terminal PVX probe. The sizes of the viral RNA species are indicated on the right.

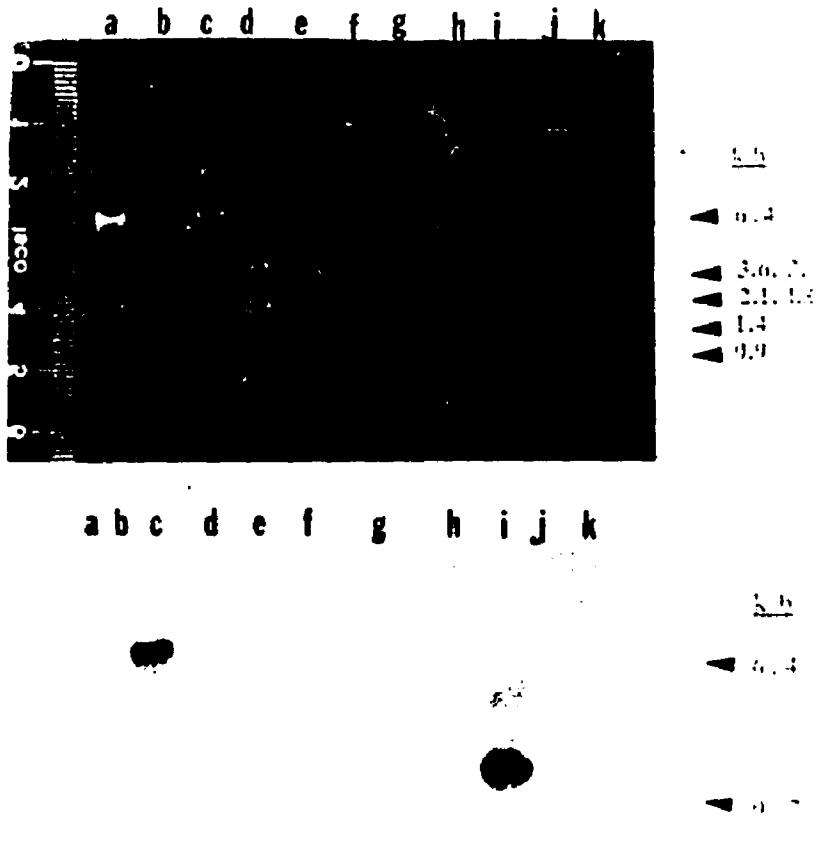
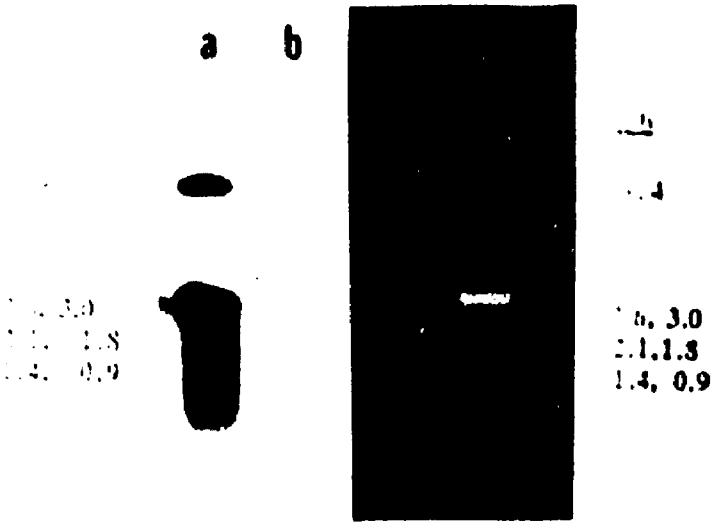
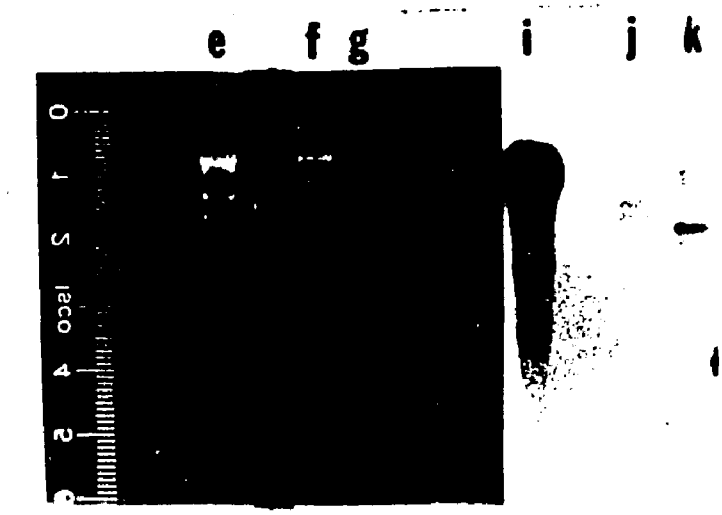

a b c d e f g h i j k
a b c d e f g h i j k

Figure 21. Northern blot analysis of PVX viral RNAs isolated under nondenaturing (top) and denaturing (bottom) conditions using a 3' terminal cDNA PVX probe. Top panel (left) : (autoradiograph of gel on right) Lane a: PVX viral RNAs, Lane b: CPMV M-RNA. A 1.0 % agarose gel containing: Lane c: PVX viral RNAs, Lane d: CPMV M-RNA, Bottom panel (left): A 1.0 % agarose-formaldehyde gel containing: Lane e: PVX viral RNAs, Lane f: pMOG 319 (EcoRI), Lane g: pMOG 115 (EcoRI). Lower panel (right side) A northern blot of the gel on the left hybridized to the 3' terminal PVX probe. Lane i: PVX viral RNAs, Lane j: pMOG 319 (EcoRI), and Lane k: pMOG 115 (EcoRI).


a
b
3.0
1.8
0.9


e
f
g
i
j
k
0
1
2
3
4
5
6
Taco

used as a 3' terminal PVX probe in Northern blot hybridization analysis. The upper panel (right side) of figure 21 shows an ethidium bromide-stained 1.0 % agarose gel containing one microgram of viral RNAs, PVX RNAs (lane c) and CPMV M-RNA (lane d). In the upper panel (left side) shows an autoradiogram of the same gel hybridized with the 3' terminal PVX probe. Six distinct bands were not clearly visible, but one distinct band corresponding to genomic PVX (lane a) was detected. Three thick bands with a high background correspond to the smaller RNA species (lane a). The high background may result from RNA degradation or the diffusion of RNA bands during hybridization. The lower panel (left side) of figure 21, shows a 1.0 % ethidium bromide-stained agarose-formaldehyde gel containing PVX viral RNAs (lane e) and two restriction fragments, pMOG 319 (EcoRI) and pMOG 115 (Xba I, Xho I) (lanes f, g). The pMOG 319 restriction fragment was added as a negative control and pMOG 115 (Xba I, Xho I) restriction fragment was added as a positive control. The lower panel (right side) shows an autoradiogram of the same gel hybridized with a 3' terminal PVX probe (pMOG 115, Xba I, Xho I). One major band corresponding to genomic PVX (lane c) was detected and several minor bands corresponding to the smaller RNA species were also detected suggesting that the smaller RNA species are 3' coterminal with PVX genomic RNA. Also detected was the pMOG 115 (Xba 1, Xho 1) restriction fragment used as the 3' terminal probe (lane k).

B. Affinity chromatographic analysis of the 3' termini of the smaller viral RNA species

An oligo dT column was used as a binding substrate to determine if the smaller viral RNA species contained a poly A tail. The presence of a poly A tail is a criterion of subgenomic RNAs if it is present at the 3' terminus of genomic RNA. BMV viral RNAs were added to the column and eluted in the void volume. BMV viral RNAs have a tRNA-like structure at their 3' termini (142). These RNAs were used as a negative control. Purified PVX viral RNAs were selected from the oligo dT column indicating that the smaller viral RNA species, like PVX genomic RNA, contained a poly A tail (figure 22).

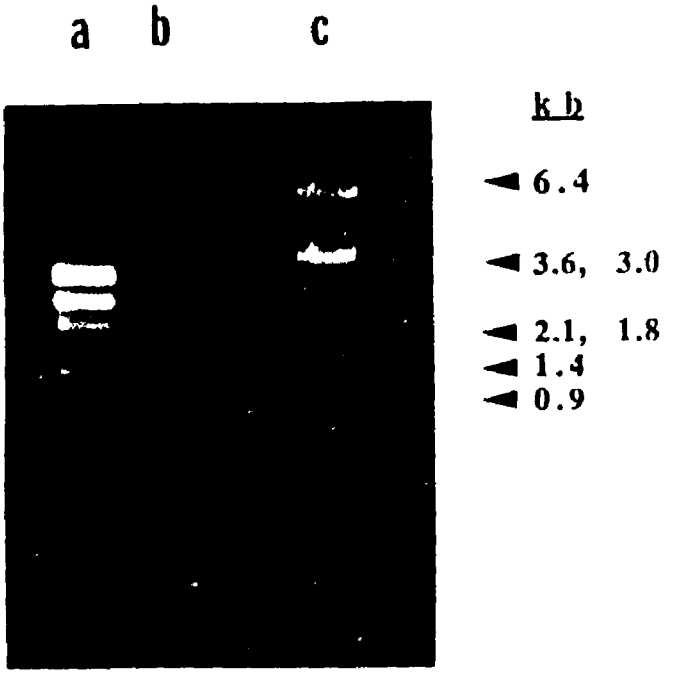
C. *In vitro* Translation of PVX viral RNAs

PVX viral RNAs were translated to characterize the products of the smaller RNA species.

1. Translation in Wheat Germ Extracts

Magnesium, potassium, and RNA concentrations were varied to determine the optimal translation reaction conditions in Promega mRNA-dependent wheat germ extracts.

Figure 22. Affinity chromatographic analysis of PVX viral RNAs. Lane a: BMV viral RNAs before affinity chromatography, Lane b: BMV viral RNA preparation after affinity chromatography, Lane c: PVX viral RNAs selected from affinity chromatography.

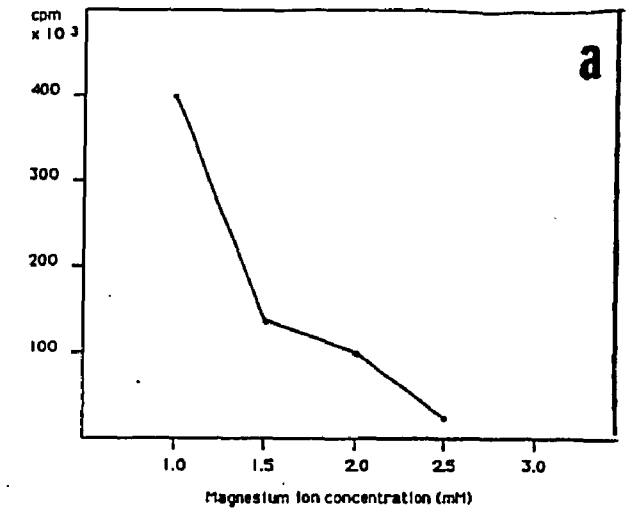

a
b
c
kb
6.4
3.6, 3.0
2.1, 1.8
1.4
0.9

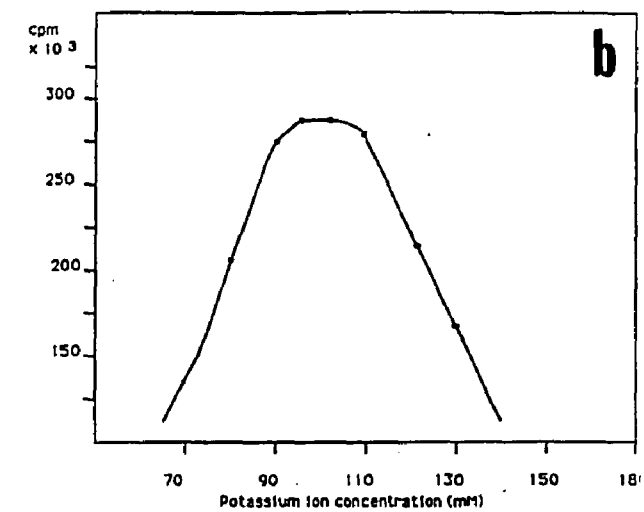
Most mRNAs are efficiently expressed within a magnesium concentration range of 1.0 to 2.5 mM (143). The magnesium concentration present in the wheat germ extract used was 1.0 mM. Magnesium concentrations added were: 0.5 mM, 1.0 mM, and 1.5 mM. Maximum incorporation of ^{35}S-methionine into proteins directed by PVX viral RNAs was very sensitive to the magnesium concentration. When magnesium was added as magnesium chloride, the optimal concentration was 1.0 mM (figure 23) at a potassium concentration of 90 mM and an RNA concentration of 100 ug/ml.

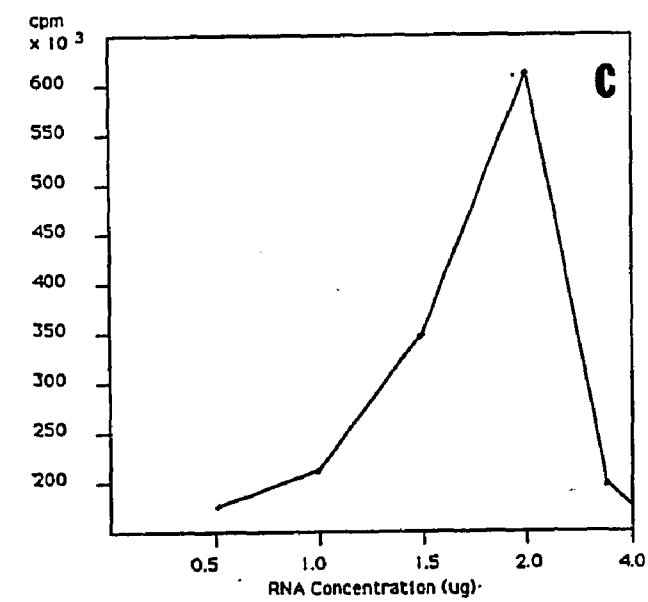
The next parameter to be varied was the potassium concentration. Most mRNAs are expressed within a potassium concentration range of 30 to 200 mM. The potassium concentration in the wheat germ extract used was 50 mM. The following potassium concentrations were added: 30 mM, 50 mM, 70 mM, 90 mM, 100 mM, 130 mM, 150 mM, 170 mM, and 200 mM. The optimum potassium concentration directed by PVX viral RNAs was not as critical as the magnesium concentration. When potassium was added as potassium acetate, the optimal concentration range was 80 to 110 mM (figure 23).

The final parameter to be varied was the RNA concentration. Final concentrations of PVX viral RNAs added to an extract containing 1.0 mM magnesium and 90 mM potassium were 25, 50,

Figure 23. Magnesium ion (a), potassium ion (b), and RNA (c) concentration dependence using PVX viral RNAs as templates in wheat germ extracts.


cpm
x 10 3
400
300
200
100
a
1.0
1.5
2.0
2.5
3.0
Magnesium ion concentration (mM)


cpm
x 10 3
300
250
200
150
b
70
90
110
130
150
Potassium ion concentration (mM)

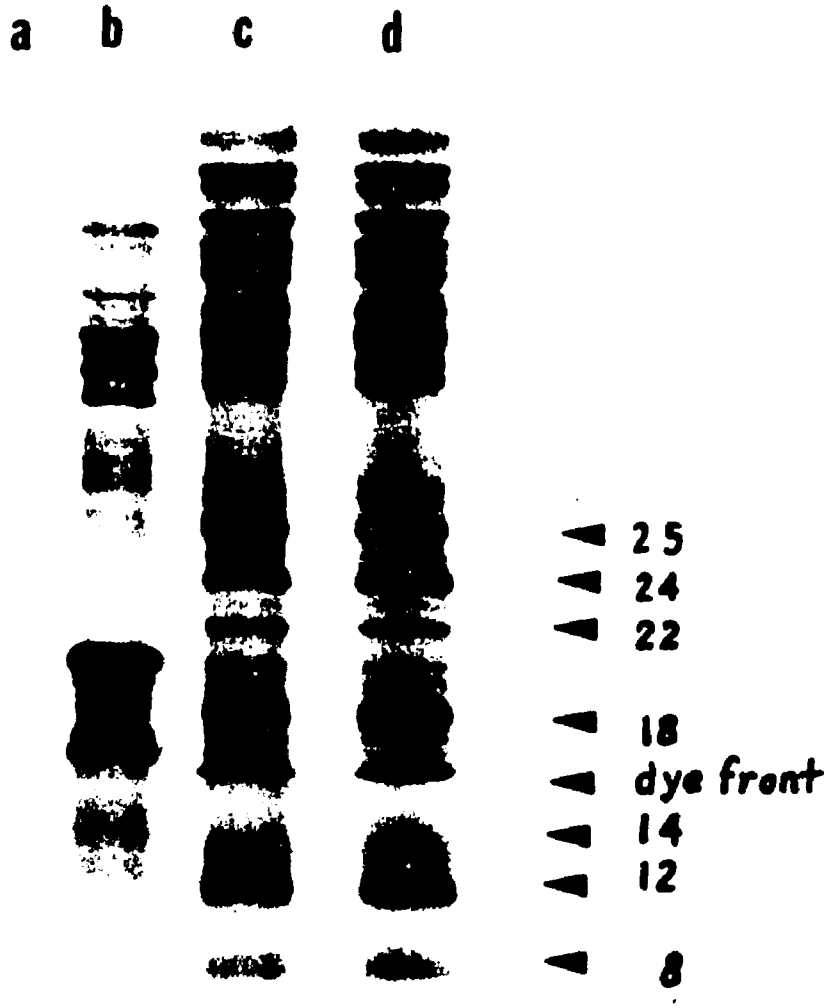

cpm
x 10 3
600
550
500
450
400
350
300
250
200
c
0.5
1.0
1.5
2.0
4.0
RNA Concentration (ug)

100, and 200 ug/ml. An optimum PVX RNA concentration of 100 ug/ml was important for maximum stimulation of ^{35}S-methionine incorporation into proteins (figure 23).

Optimized magnesium, potassium, and RNA concentrations resulted in 60 fold incorporation levels over background. Upon examination of translation products on a 12.5 % continuous polyacrylamide gel, at least 20 polypeptides were detected. The majority of the proteins had a molecular weight greater than 26,000 Da. Since the coding capacity of the largest subgenomic RNA is less than 26 kDa in mass, the larger proteins are probably products of the genomic RNA. These proteins may be products of nuclease-fragmented genomic RNA, premature termination, and internal re-initiation. Seven discrete proteins less than 26 kDa were present with molecular weights of: 25 kDa 24 kDa, 21 kDa, 18 kDa, 14 kDa, 12 kDa, and 8 kDa. Four of these proteins have masses that coincide with predicted ORFs: 25,118 (ORF 5), 24,547 (ORF 2), 12,589 (ORF 3), and 7,943 (ORF 4). Two of the remaining three proteins may be products of the 3.6 kb and 3.0 kb RNAs (figure 24).

To minimize the expression of artifactual proteins from genomic RNA, the following was tried:

Figure 24. Translation products of PVX viral RNAs programmed in wheat germ extracts. Autoradiograph of ^{35}S-methionine labeled proteins after separation on a 12.5% SDS-polyacrylamide gel. Lane a: minus RNA control, Lane b: translation products of BMV viral RNAs in wheat germ extracts, and Lanes C, D: translation products of PVX viral RNAs in wheat germ extracts.


a
b
c
d
25
24
22
18
dye front
14
12
8

a. heating the RNA template

If an RNA has extensive secondary structure, the ribosome can pause and fall off or reinitiate further down the RNA producing artifactual proteins. It is known that heating RNA to 65 degrees centigrade and quenching immediately on ice will increase translation efficiency by disrupting local secondary structure especially in G-C rich regions (118). Different preparations of PVX viral RNAs were heated prior to translation but incorporation levels and protein patterns remained the same as the unheated RNA sample.

b. addition of proteinase inhibitors

It is possible that the heterogenous polyprotein pattern may arise from protein instability. Aprotinin was added to wheat germ extracts in concentrations of 0.5 and 1.0 mM. When aprotinin was added, incorporation levels were normal, but the protein pattern was unchanged.

c. addition of RNasin

Although trace amounts of RNasin are routinely added to wheat germ extracts, increased levels did not alter the protein pattern when PVX viral RNAs were added to the altered wheat germ extracts. This is not surprising since TMV is the same size as PVX and even when low levels of RNasin are added to wheat germ

extracts only a few proteins are produced. However the sequence and secondary structure of PVX RNA may make it more vulnerable to RNases so it was tried.

d. addition of potassium

Elevated levels of potassium in wheat germ are known to promote the elongation of peptides that might terminate prematurely. The viral RNAs were incubated initially in 88 mM potassium and after a 30 minute incubation at 25 degrees centigrade, 70 mM of potassium was added. The protein pattern remained unchanged.

The different manipulations mentioned above failed to effect the protein pattern.

2. Translation in Rabbit Reticulocyte Lysate

PVX viral RNAs were used as templates in mRNA-dependent rabbit reticulocyte lysate to compare viral protein products with those in wheat germ. To optimize translation conditions for PVX viral RNAs in rabbit reticulocyte lysate, magnesium, potassium, and RNA concentrations were varied.

A typical lysate contains 1.6 to 1.8 mM magnesium (143). Since the endogenous magnesium concentration was unknown, the following magnesium concentrations were added: 0.5 mM and 1.0

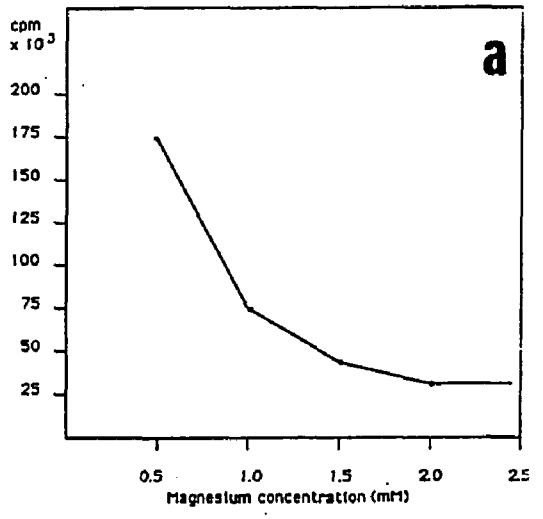
mM. The incorporation levels of ^{35} S-methionine into proteins directed by PVX viral RNAs were very sensitive to the magnesium concentration. When magnesium was added as magnesium acetate, the optimum concentration was 0.5 mM (figure 25). Other reaction conditions held constant were an added potassium concentration of 80 mM and an RNA concentration of 100 ug/ml.

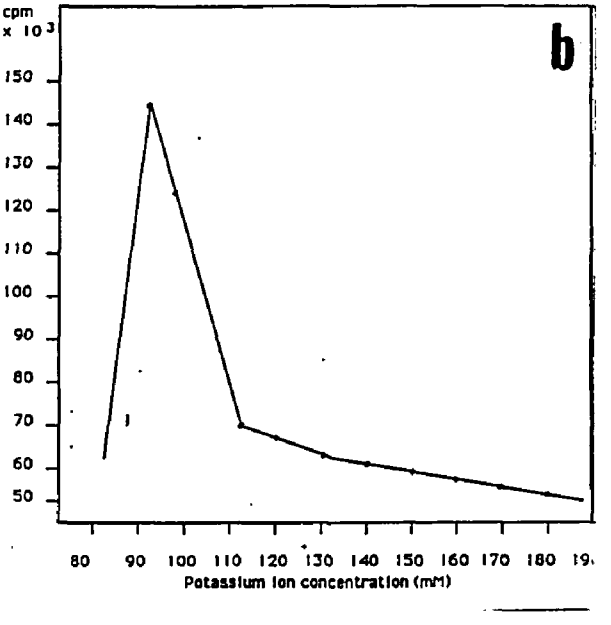
It is known that lysate usually contains 25 to 40 mM potassium (143). Since the potassium concentration was undefined, the following potassium concentrations were added: 5mM, 35 mM, 65 mM, 95 mM, 125 mM, and 175 mM. The optimum potassium concentration was important also. When potassium was added as potassium acetate, the optimum concentration was 95 mM (figure 25).

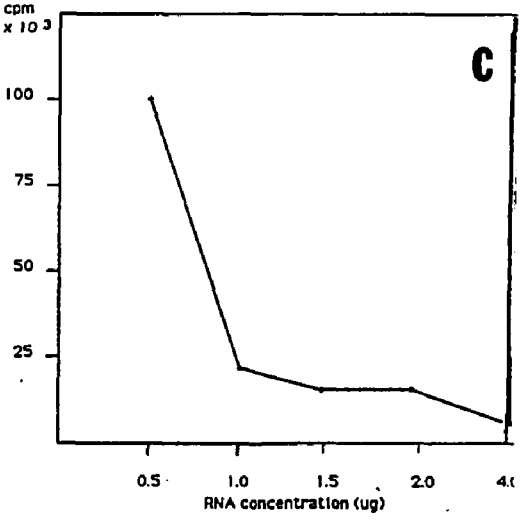
The last parameter to be optimized was the RNA concentration. At added magnesium and potassium concentrations of 0.5 mM and 95 mM, RNA concentrations of 25, 50, 100, and 200 ug/ml were tested. Maximum incorporation of ^{35} S-methionine into proteins directed by PVX RNAs occurred at an RNA concentration of 25 ug/ml (figure 25).

Upon analysis of proteins produced in rabbit reticulocyte lysate in a 12.5% discontinuous polyacrylamide gel, one polypeptide was predominant, 166,000 Da. Proteins with masses between 45,000

Figure 25. Magnesium ion (a), potassium ion (b), and RNA (c) concentration dependence using PVX viral RNAs as templates in rabbit reticulocyte lysate.


a
cpm
x 10 3
200
175
150
125
100
75
50
25
0.5
1.0
1.5
2.0
2.5
Magnesium concentration (mM)


b
cpm
x 10 3
150
140
130
120
110
100
90
80
70
60
50
80
90
100
110
120
130
140
150
160
170
180
Potassium ion concentration (mM)


c
cpm
x 10 3
100
75
50
25
0.5
1.0
1.5
2.0
RNA concentration (ug)

to 150,000 are probable products of the genomic RNA. Three additional proteins encoded by predicted ORFs were detected: 25 kDa, 24 kDa, and 12 kDa. Additional proteins of masses not predicted by ORFs include: 21 kDa, 18 kDa, and 14 kDa (figure 26).

In comparing translation products from wheat germ extracts and rabbit reticulocyte lysate, there is preferential expression of the 166 kDa protein (ORF 1) in rabbit reticulocyte lysate and 24 kDa (ORF 2), 12 kDa (ORF 3), 8 kDa (ORF 4), and 25 kDa (ORF 5) proteins in wheat germ extracts. These results are in agreement with the fact that larger mRNAs are most favorably expressed in rabbit reticulocyte lysate while smaller proteins are preferentially expressed in wheat germ extracts (143). There was good expression of the 24 kDa (ORF 2) and 25 kDa (ORF 5) proteins in rabbit reticulocyte, while the smaller proteins of 12 kDa (ORF 3) and 8 kDa (ORF 4) were barely detectable. It is interesting to note that although the 166 kDa (ORF 1) protein was not detectable intact in wheat germ extracts, there were more large polyprotein products present than in rabbit reticulocyte lysate. This is probably due to higher RNase content in wheat germ extracts (142). Additional proteins of masses not predicted by ORFs included masses of: 20 kDa, 18 kDa, and 14 kDa. These three proteins were also detected in wheat germ extracts suggesting that they are not artifacts (figure 27)

Figure 26. Translation products from rabbit reticulocyte lysate using PVX viral RNAs as templates. Autoradiograph of ^{35}S-methionine-labeled proteins after separation on a Laemmli (5% upper, 12.5 % lower) polyacrylamide gel. Lane a: low molecular weight markers, Lane b: minus RNA control, Lane c: translation products using BMV viral RNAs as templates, Lanes d-j: translation products using different PVX viral RNA preparations as templates. The upper panel is an autoradiograph exposure at 24 hours. The lower panel is an autoradiograph exposure at 72 hours.

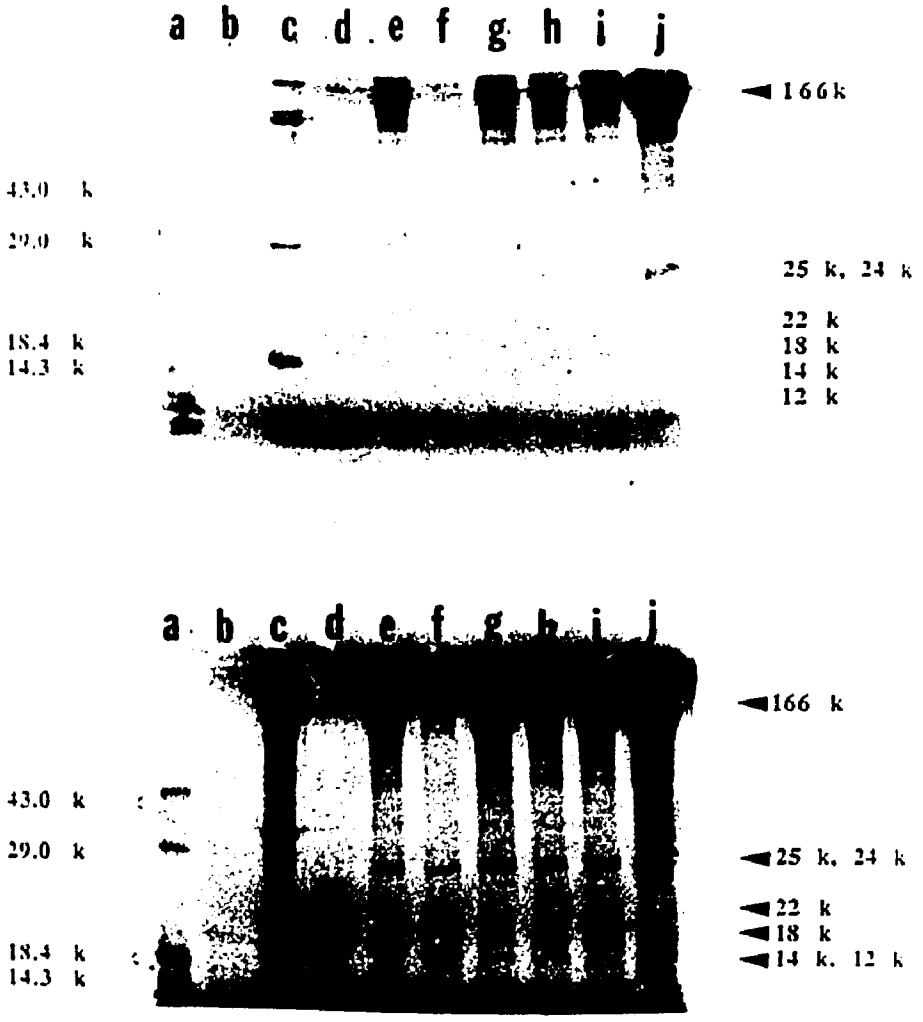
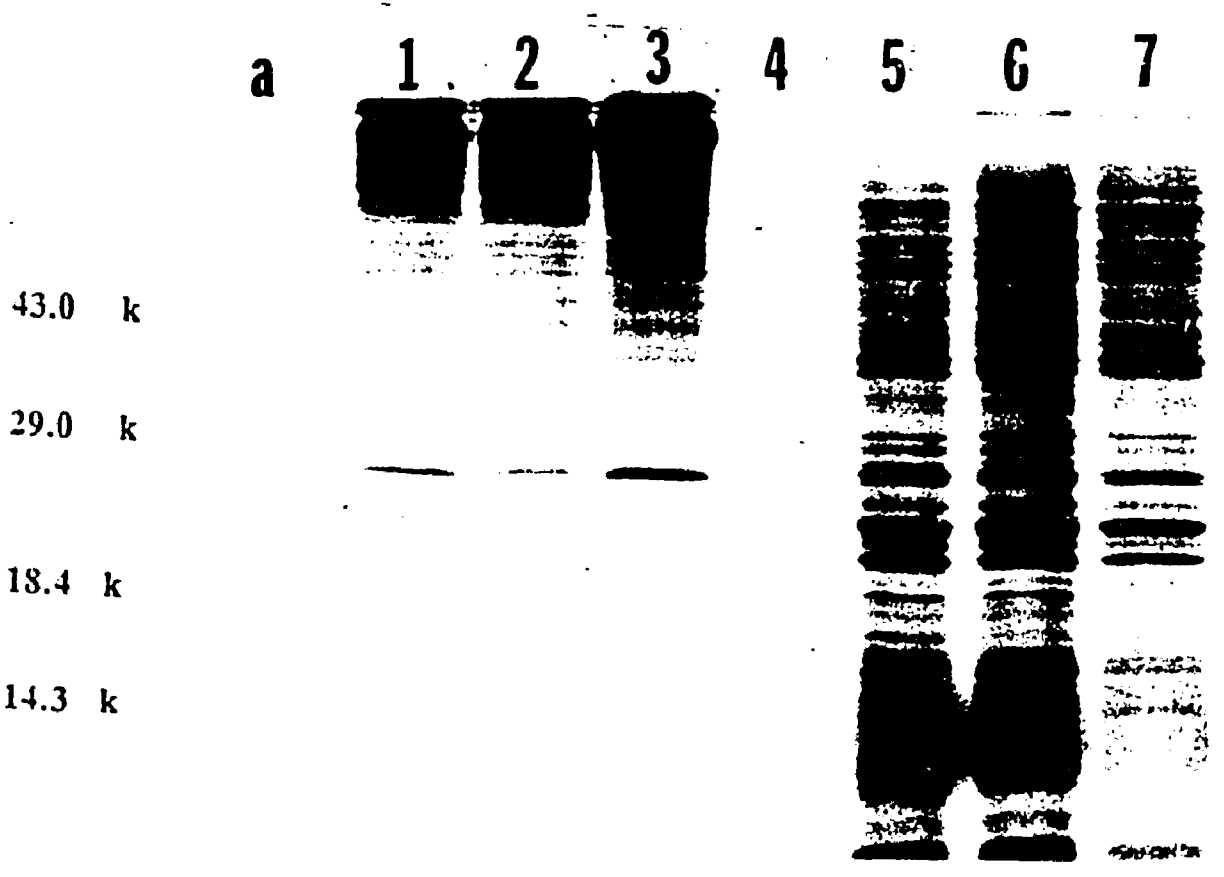

a b c d e f g h i j
166k
43.0 k
29.0 k
25 k, 24 k
22 k
18 k
14 k
12 k
18.4 k
14.3 k
a b c d e f g h i j
166 k
43.0 k
29.0 k
25 k, 24 k
22 k
18 k
14 k, 12 k
18.4 k
14.3 k

Figure 27. Comparison of translation products of PVX viral RNAs in rabbit reticulocyte lysate and wheat germ extracts. Autoradiograph of ^{35}S-methionine labeled proteins separated on a 12.5 % SDS-polyacrylamide gel. Lane a: low molecular weight markers, Lanes 1, 2, 3: translation products of PVX viral RNAs in rabbit reticulocyte lysate, Lane 4: minus RNA control, Lanes 5, 6, 7: translation products of PVX viral RNAs in wheat germ extracts.


a
1
2
3
4
5
6
7
43.0 k
29.0 k
18.4 k
14.3 k

3. Immune precipitation of viral translation products

To determine if the proteins produced in *in vitro* translaion systems programmed with PVX viral RNAs were viral proteins, extracts were treated with antisera made to peptides containing sequences to four of the five predicted proteins. Antisera to the capsid protein (ORF 5) was purchased from ATCC. Upon immune precipitation of translation products using PVX viral RNAs in wheat germ extracts and rabbit reticulocyte lysate (144), faint bands corresponding to 166 kDa, 25 kDa, 24 kDa, 12 kDa, and 8 kDa proteins were detected. However, definitive confirmation that the translation products were of viral origin could not be made.

D. Primer extension analysis to map the 5' termini of the smaller viral RNAs

Primer extension can be used to map the 5' ends of subgenomic RNAs (145). In addition, direct RNA sequencing can be performed to confirm that the smaller RNA species correspond in sequence to that derived from predicted ORFs on the genomic RNA (146, 147). The 5' ends of the predicted internal ORFs which correspond to the 2.1, 1.8, 1.4, and 0.9 kb subgenomic RNAs should show a discrete size fragment confirming that the subgenomic RNAs are not heterogenous fragments. Four internal ORFs are predicted on the genomic RNA, but six smaller RNA species were isolated from

purified virus preparations. The presence of the two additional RNA species, 3.6 kb and 3.0 kb, suggests that two unidentified ORFs may be present. The ORFs, if present, should occur near nucleotides 2800 (3.6 kb) and 3400 (3.0 kb). Multiple ORFs are detected in these regions, the carboxyl terminus of ORF 1. Therfore, two oligomers were synthesized to two uncited ORFs corresponding to the 3.6 and 3.0 kb subgenomic RNAs. Seven oligomers were synthesized containing complementary sequences to regions 20 to 30 bases downstream of the internal ORFs (beginning at the AUG), the two uncited ORFs, and the 5' most ORF. The oligomeric sequences complementary to sequences downstream of published ORFs include:

ORF	Oligomer synthesized	Location on genome
ORF 2	5'd-C C A A U A A G A U C C U G A A G A-3'	nts 4522-4540
ORF 3	5'd-G G C C A G U U A A G A C U U U U U C A C-3'	nts 5175-5196
ORF 4	5'd-G A A C A C G A A C A C C A U U G-3'	nts 5460-5476
ORF 5	5'd-C C C A G U U G A U G G A G U U G A U G G-3'	nts 5743-5764
ORF 5'	5'd-T A C C G G T T T C C A C G C G C T C-3'	nts 83-102

The oligomeric sequences complementary to sequences downstream of unidentified ORFs corresponding to 3.6 and 3.0 kb RNAs respectively include:

ORF	Oligomer synthesized	Location on genome
3.6	5'-d C A C T A T T T G T A C T T T C G G C-3'	nts 2873-2892
3.0	5'-d C A C A A G C C T T C C C A G A C C T C-3'	nts 3462-3482

As a control, an oligomer was synthesized complementary to twenty bases downstream of the 166 kDa protein ORF. All primer sequences were derived from a published PVX genomic RNA sequence. The viral RNA from which the sequence was derived was not used for these experiments. A different isolate was obtained from the American Type Culture Collection.

Attempts to extend viral RNAs separately or together as well as single-stranded total RNA preparations from PVX-infected tissue failed. Possible reasons for this failure include: (1) consistent unknown technical error, (2) the RNAs were degraded, or (3) the sequence of the primers contained mismatches with the viral RNA used and were unable to bind for extension.

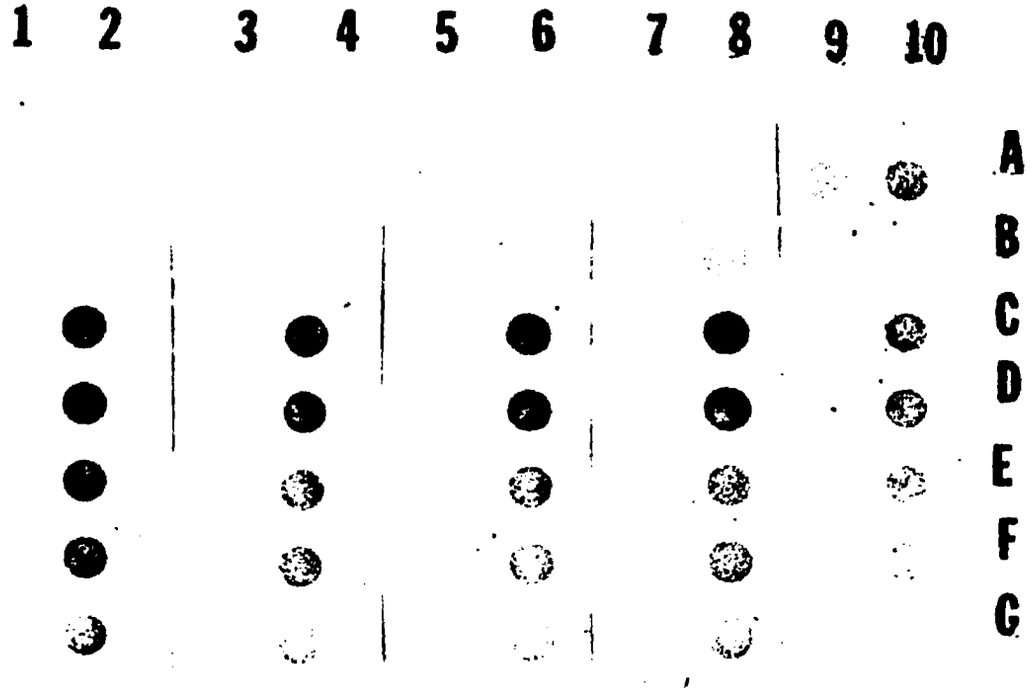
Immunoblotting analysis of cell extracts prepared from infected plants

A. Dot blot hybridization analysis to determine if the predicted ORFs are functional *in vivo* from PVX-infected leaf tissue

Since the Northern and *in vitro* translation experimental results discussed earlier were suggestive but not definitive, Western analysis was chosen to determine the number of viral proteins expressed *in vivo*. Mouse antisera was made to four synthetic peptides from predicted amino acid sequences derived from the genomic RNA sequence (36). The antigen or viral proteins were prepared from PVX-infected leaf tissue isolated 5, 7, 9, 11, and 13 days post inoculation on tobacco plants (see materials and methods). Mock-inoculated tobacco was used as a control and isolated at the same time intervals mentioned above. The leaf tissue was isolated at different time intervals since the viral proteins may be in highest titers at different times post inoculation.

Dot blot western results indicated positive serological recognition with infected crude extracts but not healthy mock-inoculated controls to all the antisera prepared (figure 28). In columns 1, 3, 5, and 7, samples in row A show no reaction. This row was a buffer control. In the same columns and rows B through G, no reactivity was detected. These rows contained mock-inoculated healthy extracts isolated at days 5, 7, 9, 11, and 13. In columns 2, 4, 6, and

Figure 28. Analysis of dot blot hybridizations. Columns 1, 3, 5, 7 Row A: buffer control, Columns 9, 10, Row A and Column 10, Row B: purified virus preparations. Column 1, Rows B, C, D, E, F, G: healthy extracts from mock-inoculated tobacco plants at 3, 5, 7, 9, 11, and 13 days post inoculation. Columns 2, 4, 6, 8, 10 , Row A: purified peptide for antisera to 166 kDa, 24 kDa, 12 kDa, and 8 kDa proteins and buffer control, Columns 2, 4, 6, 8, 10, Rows B, C, D, E, F, G: PVX-infected leaf extracts from tobacco plants at 5, 7, 9, 11, and 13 days post inoculation.


1 2 3 4 5 6 7 8 9 10
A
B
C
D
E
F
G

8, samples in row A show a positive response. This row contains purified peptides from ORFs 1, 2, 3, and 4. In these same columns, samples in rows B through G contain total protein extracts from PVX-infected leaves isolated 5, 7, 9, 11, and 13 days post inoculation. In column 9, samples in row A show a positive reaction. This row contains purified virus. Samples in rows B through G contain healthy protein extracts at 5, 7, 9, 11, and 13 post mock-inoculation. In column 10, samples in row A show no reactivity. This row is a buffer control. In column 10, samples in rows B through G show a positive reaction. These rows contain PVX-infected total protein extracts isolated at 5, 7, 9 , 11, and 13 days post inoculation. These results indicate that the predicted ORFs are expressed *in vivo*. This result also suggested that at least four subgenomic RNAs are produced during replication.

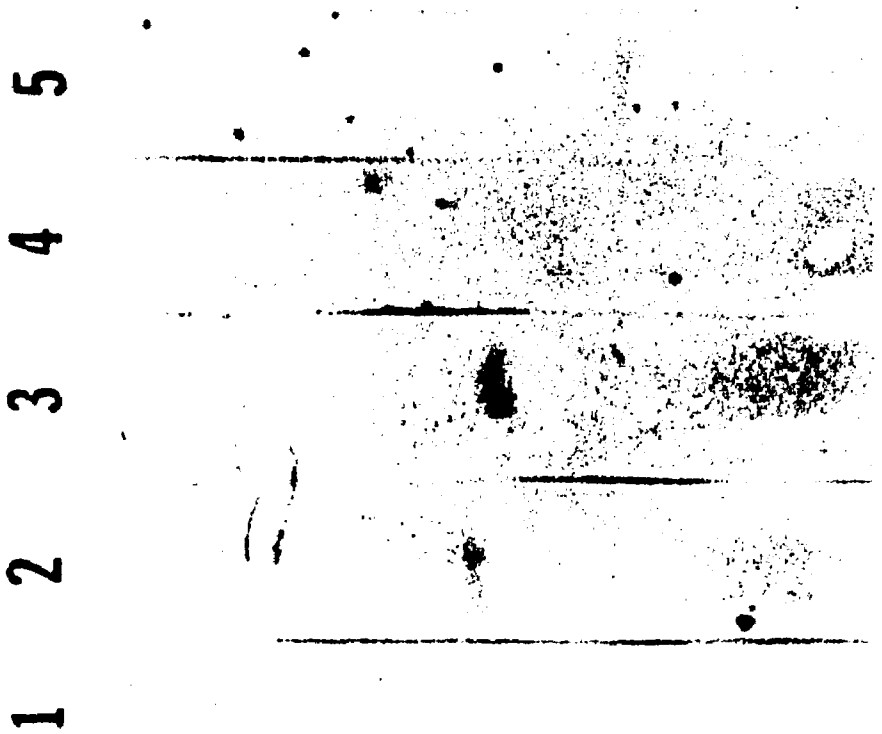
B. Western analysis protocol used to determine the number of proteins produced *in vivo*

The dot blot western confirms that the five predicted ORFs are expressed *in vivo*, however, six subgenomic RNAs are reported to exist (68). Therefore, if all subgenomic RNAs are functional, seven proteins should be expressed. A western was done to determine the total number of viral proteins produced during PVX replication. Crude extracts from 5 days post inoculation were used since the dot blot western experiment indicates that all five antisera reacted strongly.

If more than five proteins are detected, this would suggest the possible existence of internal ORFs within the reported ORFs. Since two RNAs of sizes 3.6 and 3.0 were detected, two additional ORFs may occur within ORF 1.

Western analysis re-confirmed the dot blot western results when five protein bands were detected, one to each of the five antisera. However, since two ORFs are suspected to occur within ORF 1, two different transfer times were done. The first transfer time was 5 hours and the 166 kDa protein was detected. The second transfer time was 3 hours. Proteins from 10 to 90 kDa should transfer in this time interval. Two faint bands reacted with the antisera made to the 166 kDa protein suggesting that two additional ORFs not cited in the literature are present within ORF 1 and encode proteins of approximate molecular weights 18 kDa and 14 kDa respectively. Positive results detecting two faint bands with antisera prepared to the 166 kDa protein (lane 1), the coat protein (25 kDa protein) (lane 2), 24 kDa (lane 3), 12 kDa (lane 4), and 8 kDa (lane 5) proteins are shown in figure 29.

Figure 29. Western analysis of PVX viral proteins produced *in vivo*. Lane 1: 166 kDa antisera, Lane 2: coat protein antisera, Lane 3: 24 kDa protein antisera, Lane 4: 12 kDa antisera, and Lane 5: 8 kDa antisera.


1
2
3
4
5

CHAPTER IV. DISCUSSION

PVX has been studied for at least forty years. It has been defined as flexible, rod-like particles of uniform size. The recent observation of seven particle sizes is confusing at first glance. Recent literature mentions only one particle size, but show no electron micrographs corroborating this (36). However, journal articles from 1946 (54) and throughout the 1960's (48, 49, 53) contain electron micrographs of PVX which show at least five different smaller particle sizes. The size distribution of the smaller particles was determined, but the authors failed to investigate their possible role in PVX replication. The smaller particles were simply regarded as fragmentation artifacts. Evidence in this study suggests that PVX contains multiple encapsidated subgenomic RNAs not necessary for infectivity. This is not a novel phenomenon as the cowpea strain of TMV is such an example (90).

The fact that recent literature fails to report different sizes of PVX is difficult to reconcile with the observations made in this study. Six different purification procedures were used to isolate PVX. Some were specifically designed for PVX (36, 50, 51, 55) while others were designed for other rod-like plant viruses (51, 114). Upon completion of each purification procedure, seven discrete modal particle sizes were detected by electron microscope.

SSEM examination of crude extracts from infected leaves showed a similar distribution of particle sizes. Thus the SSEM studies support the notion that small particles are not artifacts resulting from fragmentation of the viral genome during purification.

In PVX-infected leaf tissue, six subgenomic mRNAs were reported in total RNA preparations (68). If PVX protein expression is directed by subgenomic mRNAs in addition to the full-length genomic RNA as recent experiments suggest, there could be six corresponding particle sizes in addition to the full-length virus particle if the assemby initiation site is located at the 3' end. In this investigation, electron micrographs show six different particle sizes which is consistent with the number of RNAs. However, both are inconsistent with the genomic organization of PVX RNA, which is reported to contain five protein sequences (36). The discrepancy of two additional RNAs could be explained by one or more RNAs being cleavage products, electrophoretic artifacts or degradation products. The consistent presence of two additional virus particle sizes, and the two RNAs they are presumed to contain, might also mean that more than five proteins are encoded in the virus genome. Alternatively, the two additional particles could be truncated versions of full-length genomic RNA or subgenomic RNAs which are efficiently encapsidated (defective interfering particles). This phenomenon was found to be the case with the isometric plant virus tomato bushy stunt (148).

Dolja, Grama, Morozov, and Atabekov (68) examined single- and double-stranded RNAs from PVX purified virus preparations and total RNA extracts from PVX-infected leaf tissue. Autoradiographs revealed that purified PVX virions contain only full-length genomic RNA while six additional subgenomic RNAs of sizes 3.6 kb, 3.0 kb, 2.1 kb, 1.8 kb, 1.4 kb, and 0.9 kb were detected in infected leaf tissue. If these additional RNAs were subgenomic, then they could be present in purified virus preparations as well.

After one year of trying to detect smaller RNA species from partially purified virus preparations, PVX virus was purified by a butanol/Triton-X/PEG method (114). Upon electron microscope examination, the virus was in aggregated form. The virus concentration was lower, 0.1 - 1.0 ug/ul instead of 10 - 20 ug/ul. Upon extraction of viral RNA by several different methods (36, 136, 137, 138), six subgenomic RNAs were observed. The subgenomic RNAs corresponded to sizes reported by Dolja et al. (68) from total RNA isolated from infected leaf tissue. It was concluded that the lower virus concentration coupled with an increased SDS concentration facilitated partial disaggregation of virion clumps allowing detection of the subgenomic RNAs. In addition, we confirmed the presence of seven viral RNAs of appropriate sizes were detected upon extraction of total RNA from PVX-infected leaf tissue.

The smaller virion RNAs detectable were smeared. This could be due to ribonuclease contamination or incomplete capsid protein digestion. Upon virus purification (114), a green color was still present (this was dependent upon the host used) after the initial homogenization with butanol. The virus purification method was further modified to reduce ribonuclease contamination. Proper virus purification and RNA extraction methods are important in the detection of the smaller RNAs. The smaller RNAs appear to be easily degraded.

With the detection of six smaller RNAs from purified virion preparations, it was important to determine if these RNAs had characteristics of plant viral subgenomic RNAs. The first criterion examined was to determine if the smaller RNAs shared sequence homology with the 3' end of genomic RNA by Northern blot analysis.

Examination of PVX viral RNAs by Northern blot analysis was difficult. Clear sharp bands detecting subgenomic RNAs have not been published. Most published potexvirus northern hybridizations show high background levels with the putative subgenomic RNAs from total RNA extracts of PVX-infected leaf tissue barely detectable above this high background. The high background is attributable in part to high levels of RNA degradation. Another reason may be that routine hybridization protocols used for most

viral RNAs is not optimized for the detection of potexviral subgenomic RNAs. Experiments designed to determine if the smaller RNAs shared seqeunce homology with the 3' end of PVX genomic RNA proved to be difficult. Inconsistent number and intensity of bands were seen when various restriction fragments of the genomic 3' end were used as probes. The probe might have had a tendency to hybridize with itself making hybridization results inconsistent. Different restriction fragments from a clone prepared to a specific viral RNA show different hybridization efficiencies along the genome, especially at the 3' end (Mackie, personal communication). The pMOG 115 clone containing the 3' end of PVX used for these experiments was not made from the PVX virus isolate used throughout this project.

Theoretically, if an identical 3' sequence is present in the genomic and smaller RNAs and the RNAs are present in equal intensity, then bands should appear at the same time and in equal intensity. This was not the case when PVX viral RNAs were probed with a 2.8 kb Xba I, Xho I fragment at the genomic 3' end. A band for the genomic RNA would appear within 24 hours with great intensity, but faint bands for the smaller RNAs did not appear until 2 to 5 days later. If the smaller RNAs are genomic fragments, then they should appear at the same time and in equal intensity as the genomic. The fact that in some experiments the genomic RNA did not light up intensely, but the smaller RNAs did confuses matters

more. The protocol used was probably not the cause of irreproducibility. The only inconsistent factor in this experiment was that the virus was inoculated on different host species of different ages. An inhibitor isolated from the plant in the form of carbohydrate, fatty acids, double-stranded RNAs, oxidized thiols, or fragmented viral RNAs might be present in some of the RNA preparations. The amount of inhibitor might vary with the host and its age. This would explain why hybridization of the genomic RNA was variable as well. Other reasons for erratic Northern blot hybridization results are (1) the subgenomic RNAs did not transfer as well as the genomic RNA to the membrane, (2) the secondary structure of the subgenomic RNAs was more extensive and the denaturing conditions were ineffective in completely extending the smaller RNAs, or (3) the smaller RNAs did not bind as effectively to the membrane and diffussion from the membrane occurred during hybridization.

Another criterion examined to determine if the smaller RNAs isolated from purified virus preparations were authentic viral subgenomic RNAs is the presence of a poly A tail. This is a valid criterion only if the genomic RNA has a poly A tail. PVX RNAs extracted from partially purified virus preparations were selected by affinity chromatography using oligo dT. This result confirms an earlier report in which PVX subgenomic RNAs isolated from total RNA extracts from PVX-infected tissue were found to contain poly A tails (68).

A third criterion of a subgenomic RNA is its ability to direct translation. *In vitro* translation of viral RNAs is not yet routine. Wheat germ extracts and rabbit reticulocyte lysate do not consistently support protein synthesis from all viral templates. Optimal requirements for the expression of all virus proteins from PVX genomic RNA are still unknown. Since PVX has been studied for over forty years, and detection of all encoded viral proteins has been elusive, this might suggest that the wheat germ extracts or lysate are deficient in one or more factors necessary for successful translation of PVX genomic RNA. Alternatively, extensive secondary structure of viral RNA might occlude the ribosome binding sites preventing expression of all of the unidentified proteins. The predicted secondary structure of PVX RNAs at the 5' end, beginning at AUG, has extensive base pairing (36) (figure 30). The last 1500 bases containing the coat protein gene also has extensive base-pairing (figure 31). The 180 kDa and 145 kDa proteins derived from PVX genomic RNA (57) are probably due to an (a) impure RNA preparation, or (b) aberrant internal initiation on the viral template. This conclusion is supported by a paper in which PVX RNA was sequenced (36). The RNA encodes only one high molecular weight protein of 166 kDa. It is possible, that the two high molecular weight proteins seen are readthrough and premature termination products expressed from the full-length genomic RNA. However, these proteins could be electrophoretic variants of the 166 kDa protein.

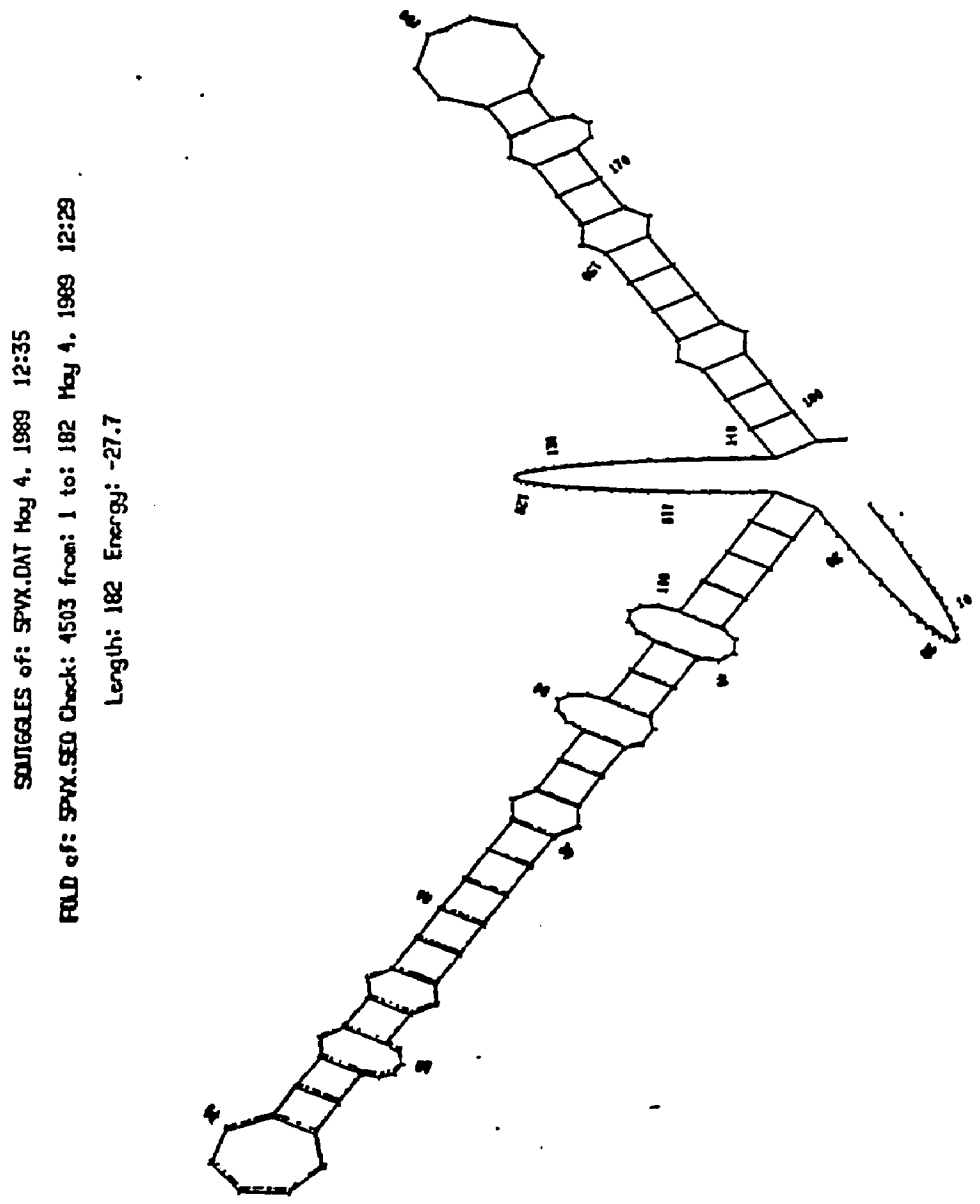


Figure 30. Predicted secondary structure of the 5' terminus of potato virus X RNA. The Squiggles computer program was used.

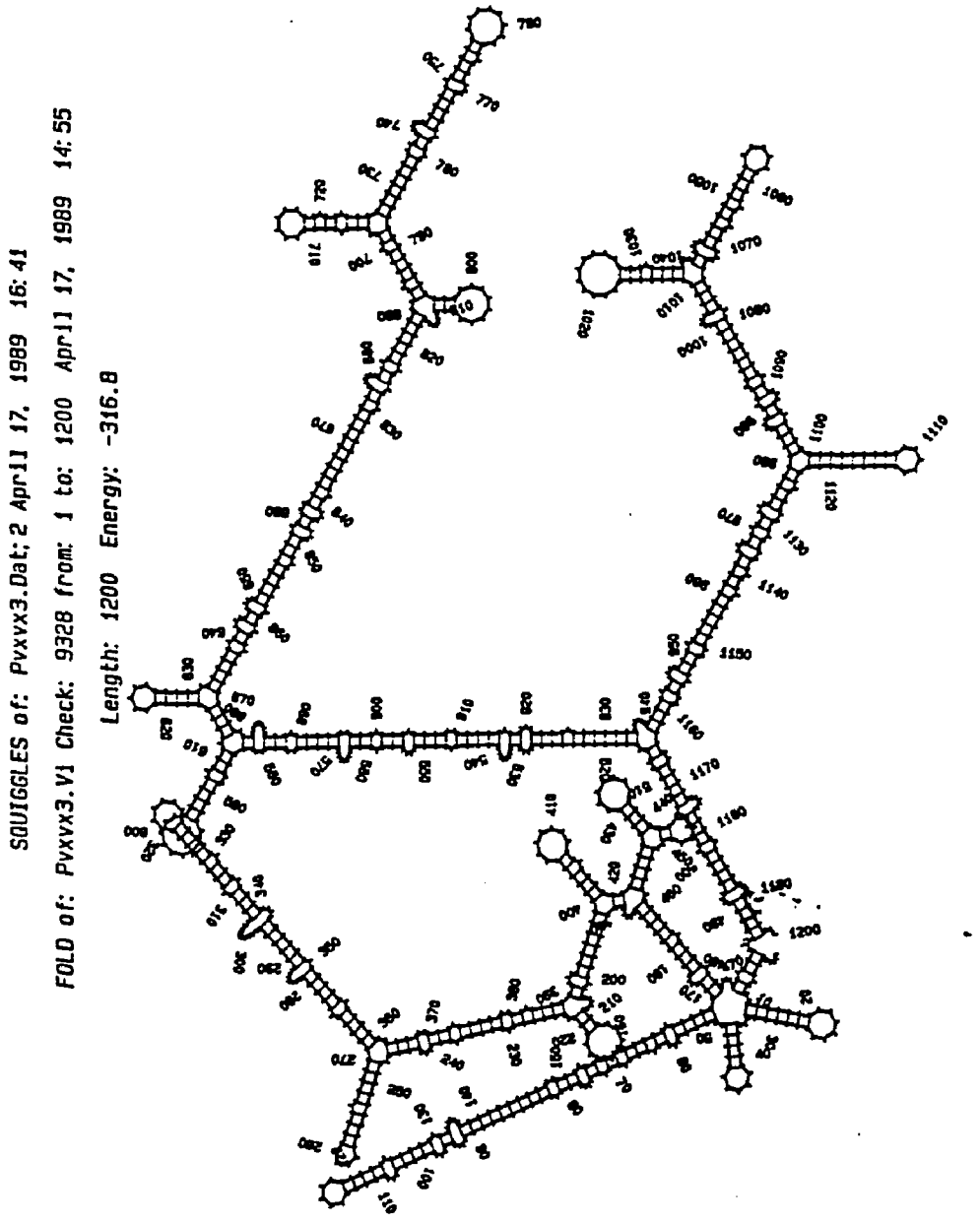


Figure 31. Predicted secondary structure of the 3' terminus of potato virus X RNA. The Squiggles computer program was used.

Initial attempts to detect proteins in wheat germ extracts and rabbit reticulocyte lysate using PVX viral RNAs as templates failed. The integrity of viral RNAs were routinely checked on an agarose gel prior to their placement in wheat germ extracts or rabbit reticulocyte lysate. The seven viral RNA species were discrete, however, there was ethidium bromide background between the RNA bands suggesting that indiscrete degradation products were present. It is important to mention that additional RNAs of 5.4 kb, 1.6 kb, and three RNAs less than 0.9 kb were present more than once. The RNAs present most often and in highest concentration were the three RNAs less than 0.9 kb. The additional RNAs could be satellites or defective interfering particles. The additive sizes of the smaller RNAs do not equal the full-length RNA, however, different RNA sequences of comparable size could co-migrate so they could still be genomic degradation products. The presence of these RNAs could have interfered with viral RNA messenger activity.

The first translation protocol used which resulted in incorporation using PVX viral RNAs was Pelham and Jackson (150). Conditions in this protocol were optimal for the expression of some potexviral coat protein RNAs. Only a two to three fold rise in ^{35} S-methionine incorporation over backgroun was obtained. No viral proteins were observed upon autoradiographic examination. Several other protocols (151, 152) were tried with similar results. One protocol (114), however, showed little endogenous protein

expression and seven proteins were seen. These proteins were preseumed to be PVX viral proteins, but subsequent attempts to repeat this result failed.

As RNA extraction protocols were improved for the detection of viral subgenomic RNAs, the incorporation of ^{35}S-methionine in the different lysate preparations were one-half the control. This result might suggest that the smaller RNAs were fragments, however, the coat protein RNA was separated from genomic RNA and *in vitro* translation results showed no incorporation. Perhaps the smaller RNAs were degraded even though they migrated as sharp bands in an agarose gel. Alternatively, an inhibitor might be present in the virion RNA preparation. Confirmation of this possibility was indicated when the purified virion RNA preparation was added to a control RNA, BMV RNAs, resulting in a decrease in incorporation as compared to the control RNA alone. In later experiments, RNA preparations showed high incorporation levels.

Interpretation of *in vitro* translation products produced by a large plant viral RNA can be difficult. It is not uncommon to detect heterogenous sizes of polypeptides. In addition, artifacts can appear consistently as discrete bands. Rabbit reticulocyte lysate is generally chosen for the translation of larger mRNAs, while wheat germ is best suited for the expression of smaller mRNAs. Wheat germ can translate preparations of mRNAs containing oxidized

thiols, and double-stranded RNAs inhibitory to rabbit reticulocyte lysate. However, wheat germ extracts contain higher levels of RNases. Large RNA fragment easily in such an enviornment resulting in the production of heterogenous proteins derived from premature termaination and internal initiation (118).

The molecular weights of five proteins synthesized by PVX viral RNAs in recent experiments of this study in wheat germ extracts and rabbit reticulocyte lysate correspond to predicted ORFs mentioned in the literature (37). Two other proteins synthesized may correspond to uncited ORFs. Western analysis confirms that at least five proteins synthesized *in vitro* are produced *in vivo*. The western is also suggestive that two additional proteins produced *in vitro* are also produced *in vivo*. The western needs to be repeated since the two smaller proteins detected using antiserum made to a peptide containing sequences to the 166 kDa protein were faint making definitive conclusions impossible at this time.

Despite erratic experimental results that were common throughout this investigation, this is the first report of six smaller RNA species being detected upon extraction of purified PVX virus preparations by ethidium bromide staining. *In vitro* translation results in wheat germ extracts and rabbit reticulocyte lysate suggest that the smaller RNAs have messenger activity. The smaller RNAs contain a poly A tail and are 3' coterminal with genomic RNA,

but share no homology at the 5' end. This is also the first report of the detection of viral proteins produced *in vivo* using virus-specific peptides made to ORFs 1, 2, 3, and 4. The presence of three additional proteins in *in vitro* translation studies and two additional smaller RNAs, 3.6 kb and 3.0 kb, suggest that two unidentified ORFs may exist within ORF 1.

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VITAE

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DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Mary Price

Major Field: Biochemistry

Title of Dissertation: Examination of Potato Virus X: their number, their coding properties, and their possible encapsidation.

Approved:

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

Date of Examination:

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